Hydrocolloid Applications

Gum technology in the food and other industries

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5 Pectins

5.1 Introduction

The natural polymers (structural materials) found in all land plants are termed pectins (Braconnot, 1825). Like starch and cellulose, pectin is a structural carbohydrate (Christensen, 1986). It was discovered in the 18th century (Vauquelin, 1790), and Braconnot was the first to characterize it as the active fruit component responsible for gel formation. He also suggested the word 'pectin', which originates from a Greek word meaning 'to congeal or solidify'.

Commercially, pectin is extracted from citrus peel or apple pomace. The commercial isolation of pectins from suitable plant material began early in the 20th century and has been developing ever since. Pectic substances are integral structural components of the cell and play an important role as cementing material in the middle lamellae (Fig. 5.1) of primary cell walls (Christensen, 1986). The many reviews and comprehensive texts on pectin are valuable sources for the reader (Kertesz, 1951; Doseburg, 1965; Pilnik and Zwiker, 1970; Christensen and Towles, 1973; Pedersen, 1980; May, 1992; Sakai *et al.*, 1993). The release of pectin involves acidic extraction and isolation by precipitation, followed by drying to obtain a powder with standard properties. Ultrasound has been suggested to intensify pectin de-esterification (Panchev *et al.*, 1994). Pectins are normally dried to less than 10% water content. The product is kept in a vapor-tight package under cool, dry conditions. Commercial pectins usually have particle sizes of ~ 0.25 mm and a low density, $\sim 0.7\ g\ cm^{-3}$. Commercial pectins include mainly polymerized galacturonic acid that has been partly esterified with methanol (Rolin and De Vries, 1990). The percentage of the partially esterified portion of polymerized galacturonic acid strongly influences the functional properties of the pectin, and pectins with both low and high ester contents are sold. At low pHs pectins with high ester contents with the addition of enough sugar create fruit-system gels (Rolin and De Vries, 1990).

Pectin is used as a gelling agent in traditionally manufactured fruit-based products, especially jams and jellies. The heat stability of pectin under acidic conditions makes it an ideal candidate for the conditions occurring when texturization or stabilization are required in acidic food systems. Home-made jam-making is based on the fruit pulp's ability to form gels when boiled with sugar: the natural pectin content in the pulp is responsible for the gelation. Commercial jam processing adds already produced pulp,

Exhibit A

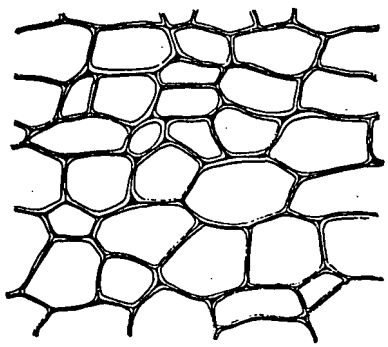

Figure 5.1 The middle lamella seen as a solid black mass between the cells of an unripe apple (× 1350), adapted from *The Pectic Substances* by Z.I. Kertesz (1951), Interscience Publishers, New York.

yielding more uniform preparations. High-ester pectins can form gels at low pH when sufficient amounts of sugar are added (thereby reducing water activity in the system). Low-ester pectins create gels in the presence of calcium ions via a different mechanism. The increasing amounts of pectins produced are now utilized outside their traditional industry as part of the confectionery industry, as stabilizers in the milk industry, and for pharmaceutical purposes.

5.2 Nomenclature

Pectin and pectic substances are heteropolysaccharides consisting mainly of galacturonic acid and galacturonic acid methyl ester residues (Christensen, 1986). To obtain uniform definitions in this area, the American Chemical Society adopted a revised nomenclature for pectic substances (Baker *et al.*, 1944) as follows.

Pectic substances are those complex colloidal carbohydrate derivatives that occur in or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units, which are thought to exist in a chain-like combination. The carboxyl groups of polygalacturonic acid may be partly esterified by methyl groups and partly or completely neutralized by one or more bases (Christensen, 1986).

Protopectin is the water-insoluble parent pectin substance that occurs in plants and which, with restricted hydrolysis, yields pectin or pectinic acid.

Pectinic acids are the colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Pectinic acids, under

suitable conditions, are capable of forming gels in water with sugars and acid, or if suitably low in methoxyl content, with certain ions. The salts of pectinic acids are either normal or acid pectinates.

Pectin (or pectins) are those water-soluble pectinic acids of varying methyl ester content and degree of neutralization that are capable of forming gels with sugar and acid under suitable conditions.

Pectic acid is a term applied to pectic substances composed mostly of colloidal polygalacturonic acids and essentially free of methyl ester groups.

Protopectinase is the enzyme that converts protopectin into a soluble product. It is also called pectosinase or propectinase (Christensen, 1986).

Pectinesterase (PE) or pectinmethylesterase is the enzyme that catalyses the hydrolysis of the ester bonds of pectic substances to yield methanol and pectic acid. The name *pectase* does not indicate the nature of the enzyme action and has, therefore, been replaced by these more specific names.

Polygalacturonase (PG) or pectin polygalacturonase is the enzyme that catalyses the hydrolysis of glycosidic bonds between de-esterified galacturonide residues in pectic substances.

Pectinase is frequently used to designate the glycosidase as well as pectic-enzyme mixtures (Baker *et al.*, 1944; Christensen, 1986).

A modern definition of pectin takes into consideration the low methyl ester content and the amidated pectinic acids as follows: pectin is a complex, high-molecular-weight polysaccharide consisting mainly of the partial methyl esters of polygalacturonic acid and their sodium, potassium and ammonium salts. In some types (amidated pectins), galacturonamide units further occur in the polysaccharide chain. The product is obtained by aqueous extraction of appropriate edible plant material, usually citrus fruit and applies (Christensen, 1986).

5.3 Structure

Commercial pectins are composed mainly of polymerized, partly methanol-esterified (1–4)-linked α-D-galacturonic acid. The pectin molecule can contain 200–1000 linked galacturonic acid units. In some pectins, the methyl ester groups are partially replaced by amide groups, to a maximum of 80% (Fig. 5.2). During extraction, only part of the pectin molecules can be extracted by non-degradative means, whereas dilute acids are generally used

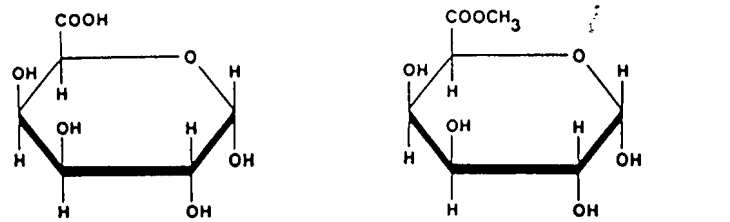

Figure 5.2 Principal units in the pectin molecule.

(Rolin and De Vries, 1990). Therefore, the structure of the resultant pectin differs greatly. About 5–10% of the galacturonic acids are neutral sugars such as galactose, glucose, rhamnose, arabinose and xylose. They can be bound to the galacturonate main-chain, be inserted into the main-chain (rhamnose) or be a part of contaminating polysaccharides (glucans and xyloglucans). Pectins from apple, citrus, cherry, strawberry, carrot, pumpkin, sugar beet, potato, onion and cabbage have the same neutral sugar composition (Amado and Neukom, 1984; Guillon *et al.*, 1986; Rolin and De Vries, 1990), in contrast to pectins from mountain pine pollen, Japanese kidney beans and duckweed, which contain large amounts of xylose or apiose (Mascaro and Kindell, 1977; Matsuura, 1984). Bacterial enzymes can be used to extract pectin from pumpkin and sugar beet (Matora *et al.*, 1995). Information on the characterization of pectic substances from selected tropical fruits such as orange, lime, banana, mango, avocado, pawpaw, cashew apple, star apple, tomato and guava, in terms of their gelation properties, can be found elsewhere (Nwanekezi *et al.*, 1994).

X-ray diffraction studies performed on dried fibers to study the structure of pectin indicated that the galacturonan backbone forms a right-handed helix, with three galacturonic acid units in C_1 conformation as the repeating sequence, corresponding to a repeat distance of 1.34 nm (Palmer and Hartzog, 1945; Walkinshaw and Arnott, 1981a). Morris *et al.* (1982) suggested that gel formation with calcium involves polygalacturonic acid sequences with a 2_1, ribbon-like symmetry. Upon drying the gel, however, the 3_1 helical symmetry is restored via polymorphic phase transition. Commercial pectins contain lower amounts of neutral sugars relative to pectin extracted under mild conditions. A large proportion of these sugars is 1,2-bound rhamnose present in the galacturonan backbone. However, rhamnose's distribution along the pectin chains has not yet been fully elucidated (Christensen, 1986). The length of polygalacturonate sequences between rhamnose interruptions has been suggested to be fairly constant, corresponding to ~25 residues (Powell *et al.*, 1982). Analysis of similar sequences with 20 to 30 degrees of polymerization (Neukom *et al.*, 1980) showed both oligomers to be almost fully made up of galacturonic acid units

with only traces of rhamnose, and galacturonan segments containing rhamnose from apple tissue. Therefore, it was concluded that cell walls can contain both a pure galacturonan-type pectin and a rhamnogalacturonan-type pectin in different regions. A molecular model of their blockwise occurrence in a few hairy regions was suggested, based on results obtained by the specific enzymatic degradation of apple pectins (De Vries *et al.*, 1982).

5.4 Sources and properties

Pectins can differ as a result of ripening and these differences can influence the efficiency of the extraction process (De Vries *et al.*, 1984; Huber, 1984; Boothby, 1983). Pectin extracted from the primary cell wall may have more branches of neutral sugars than that extracted from the middle lamella (Redgwell and Selvendran, 1986). Side-chains (neutral sugar side-chains) are distributed unevenly along the main-chain. Therefore, models describing smooth and hairy regions within pectin that has been extracted by a mild process can be deduced for pectic substances from citrus, sugar beet, cherry and carrot (Rolin and De Vries, 1990).

A description of pectic fractions from different sources can be found elsewhere (Rolin and De Vries, 1990). Substituents such as acetyl groups (in potato and sugar beet pectins) can prevent gelation. In apple and citrus, only a very low degree of acetylation is measured, and the acetyl groups may be located in the hairy regions (Vorgen *et al.*, 1986). Active pectin oligomers have also been detected in ripening tomato fruits (Melotto *et al.*, 1994). Moreover, associations of pectin with boron in the cell walls of squash and tobacco have been reported (Hu-Hi and Brown, 1994). Since pectin can come from different agricultural sources, it is not surprising that different pectins have different substituents located in different positions. Recently, pectin has been extracted from Galgal (*Citrus Pseudolimon Tan*) (Attri and Maini, 1996). The process was standardized for maximum recovery of pectin from these peels using various extractants and varying the extractant, peel ratio, extraction time, number of extractions and peel particle size (Attri and Maini, 1996). Pectin extraction from citrus peel using PG produced on whey has been reported (Donaghy and Mckay, 1994). Dried sweet whey was used as a complete medium for the production of the enzyme by the yeast *Kluveromyces fragilis*. The concentrated enzyme was then used to release pectin from the peels and apple pomace but was unable to release pectin from sugar beet pulp. Conditions for pectin extraction from orange peel were optimized with regards to enzyme concentration, water:peel ratio, temperature and duration of treatment (Donaghy and Mckay, 1994).

The most abundant substituent is the methanol ester of galacturonate residues. If apple or citrus pectins are not subjected to de-esterification, their

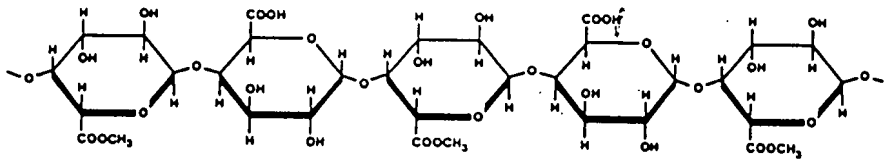

Figure 5.3 Section of a high ester content pectin molecule, with a DE ~60%.

degree of esterification (DE) is high (~70%) compared with the low DE values for pectins extracted from sunflower heads, potato, tobacco and pear (Vorgen *et al.*, 1986; Turmucin *et al.*, 1983; Pathak and Shukla, 1981). The DE is defined as the ratio of esterified galacturonic acid units to the total number of galacturonic acid units in the molecule (Fig. 5.3). These values can also be influenced by the degree of ripening of the raw material and changes in the extraction procedure. Ester group distribution depends on the source. There is evidence of random intramolecular distribution in mildly extracted apple pectins, contradicting a work which reported some regularity (De Vries *et al.*, 1983 and De Vries *et al.*, 1986, respectively). Non-random distribution has been reported in commercial pectins (Anger and Dongowsky, 1984, 1985; De Vries *et al.*, 1986). Information on de-esterification by fungal enzymes, pectin structure, conformation in solution and gels, and other properties can be found elsewhere (Kohn *et al.*, 1983, 1985; Markovic and Kohn, 1984).

The main sources of commercial pectins are citrus (lemon, lime, orange and grapefruit) peel and apple pomace. Peels are supplied for pectin production after the juice has been squeezed and the essential oils extracted. After conveying the peels to the extraction site, a water wash is used to remove as much water-soluble material as possible, other than pectin, and then extraction is begun or the peel is dried for future processing. It is not surprising to find pectin plants near plants that can supply them with the raw material directly, such as those producing apple or citrus juice, or cider. Apple pomace, which once served as the major raw material, has been replaced to a large extent by citrus peel because the latter contains 15–20% more pectin on a dry weight basis. During the Second World War, sugar beet waste (from sugar production) served as a source of pectin production. Since this pectin contains acetyl ester, other better sources are preferred. As mentioned earlier other raw materials exist (Pathak and Shukla, 1978).

5.5 Pectin manufacture

Pectin manufacturing processes are generally known (Fig. 5.4). However, variations in, or fine-tuning of, the processes, i.e. the specific conditions used, are kept confidential by the manufacturers who consider them trade secrets.

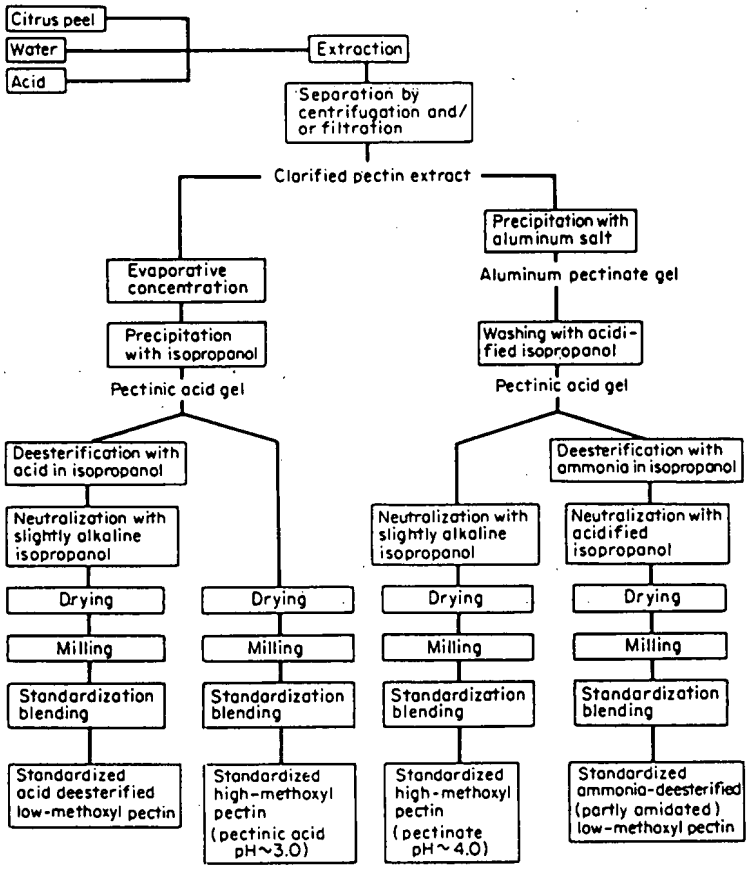

Figure 5.4 Various process routes for the manufacture of pectins. (Adapted from *Handbook of Water-Soluble Gums and Resins*, McGraw-Hill, NY, 1980, R.L. Davidson, ed.)

In general, the fresh or dried raw material (apple pomace, citrus peel and a number of other surplus materials such as sunflower bottoms and sugar beet waste) (Karpovich *et al.*, 1981) is extracted in demineralized water that has been acidified with mineral acid to give a pH of 1.5 to 3.0 (hydrochloric or nitric acid are most often used) at 70°C and for ~3 h. In the case of citrus peel, pretreatment of the peel by blanching and washing to eliminate PE activity and to remove glucosides, sugars and citric acid is common. Dried peel is stable under storage conditions, making its transport over great distances feasible. Dried citrus peel contains ~20–30% pectin. Dried apple pomace yields 10–15% pectin.

Since a certain degree of pectin de-esterification takes place during the extraction, conditions should be chosen to fit the desired product (Rolin and De Vries, 1990). Temperature, pH and time need to be carefully controlled.

Rapid-set high-ester pectins are generally extracted at temperatures close to boiling. At these high temperatures, hydrolysis of the parent pectic substances accelerates, viscosity is lowered and diffusion facilitated. This process may take less than 1 h with only minor de-esterification, whereas lower extraction temperatures and longer extraction times favor de-esterification to yield slow-set high-ester pectins or even low-ester pectins. After separating out the extract, the peels can be used as cattle feed, while the extracted liquid (viscous liquid containing 0.3–1.5% dissolved pectin) is clarified by filtration and centrifugation. At this stage, the clear pectin extract can be further de-esterified by maintaining controlled pH and temperature. The extract can be concentrated and, after preservation with sulfur dioxide, sold as 'liquid pectin'.

Pectin can be isolated by alcohol precipitation or by precipitation as an insoluble salt. With the former procedure, the pectin (unlike the water-soluble materials in the extract) precipitates out, and the alcohol is recovered by distillation. Alcohol precipitation is effected by mixing the extract with methanol, ethanol or 2-propanol. In some processes, the extract is concentrated by evaporation prior to precipitation to minimize distillation costs (Rolin and De Vries, 1990). Other alternative procedures involve pectin separation by aluminum or copper ions as an insoluble salt (Kausar and Nomura, 1982; Michel *et al.*, 1981). These metal ions can later be removed by acidified alcohol washes then a wash in alkaline alcohol to neutralize the product. The resultant alcohol-wetted pectin is pressed, dried and milled, or is de-esterified in the alcohol suspension (Rolin and De Vries, 1990).

De-esterification can be achieved with an acid or base. If ammonia is used, then some of the methyl ester groups are replaced by amide groups and the product is termed 'amidated pectin'. For pectin manufacture, as for other hydrocolloids, a blending and standardization stage is important. With the inclusion of this stage, the marketed blends exhibit very similar performance with respect to firmness of the resultant gel and the time necessary to gel high-ester pectins under predetermined constant conditions. In a similar manner, low-ester pectins are standardized in terms of their calcium reactivity.

Pectin manufacturers are located in many places worldwide. A few examples are Hercules (factories in Denmark, Germany and Florida, USA), Unipectine (France), Pektin-Fabrik (Germany), General Foods Corp. (USA) and Pectina de Mexico. Smaller pectin manufacturers are found in Switzerland, Brazil, Israel, Argentina and a few other European countries.

5.6 Commercial availability, specifications and regulatory status

Descriptions and terminology of commercial pectins exist (Doesburg, 1965). Commercial pectin is defined as the partial methyl esters of polygalacturonic

acids and their sodium, potassium, calcium and ammonium salts. The pectin is extracted from edible plant organs and no organic precipitants other than methanol, ethanol and isopropanol are used. Amidated pectins can be produced by ammonia treatment. Standardization can be achieved by dilution with sugars. Buffer salts are permitted to yield desirable setting conditions.

Commercial pectins are divided into high- and low-ester pectins in accordance with their DE values: a value over 50% is considered a high-ester pectin, values from 50% to negligible amounts define a low-ester pectin (Rolin and De Vries, 1990).

Pectate is a polymerized galacturonic acid with no or only negligible esterification. The degree of amidation (DA) is the percentage of galacturonic acid subunits that are amidated. High-ester pectins used for gel-making can be divided into rapid-set, medium-set and slow-set pectins, depending on the time necessary for solidification. The higher the DE (in high-ester pectins), the shorter the setting time. High-ester pectins are regularly standardized to 150 grade of USA-SAG, meaning that 1 part pectin can solidify 150 parts of sucrose into a jelly with the standard properties of 65° Brix (soluble solids), pH 2.2–2.4 and 23.5% SAG (indication of gel strength, see section 5.8.1).

In addition to the definitions of commercial pectins, purity is also defined by several requirements: galacturonic acid content >65%, DA <25%, loss on drying not more than 12%, acid-insoluble ash not more than 1%, alcohol residues of all kinds not more than 1%, nitrogen not more than 2.5% and sulfur dioxide not more than 50 mg kg^{-1} (Food Chemicals Codex, 1981; Anon., 1978; Anon., 1981a,b). Since pectin is an important constituent of land plants, it is consumed in significant quantities. Pectin passes unchanged (no enzymatic degradation) to the large intestine, where bacteria use it as a carbon source. However, its hydrolysation in the intestinal tract produces next to no calories (Cambell and Palmer, 1978). From a toxicological point of view, there are no limitations on its use (Anon., 1981a,b). Pectins are GRAS for use in human foods. The FDA has not issued any specific limitations or guidelines for their use in any food (Anon., 1981c). The potential dietary benefits of citrus pectin and fiber have been reviewed by Baker (1994). Other health aspects of pectin are important and have been studied by many researchers. Examples include the role of pectin in cholesterol regulation (Cerda, 1994), citrus pectin and cholesterol interactions in the regulation of hepatic cholesterol homeostasis and lipoprotein metabolism in the guinea pig (Fernandez *et al.*, 1994), the use of pectin as a fat replacer (Hoefler, 1994), oral administration of modified citrus pectin as an inhibitor of spontaneous prostate cancer metastasis in rats by inhibiting carbohydrate-mediated cell–cell interactions (Pienta and Raz, 1994), the dose response of colonic carcinogenesis to pectin and guar gum (Klurfeld *et al.*, 1994), and the effects of structural parameters of pectin on its interaction with drugs *in vitro* (Fritzsch *et al.*, 1994). Recently, a pectin-supplemented

enteral diet was reported to reduce the severity of methotrexate-induced enterocolitis in rats (Mao *et al.*, 1996). Information on the preparation and physicochemical properties of polymer complexes of benzimidazolyl-2-methylcarbamate and apple pectin can be found (Khalikov *et al.*, 1995), and a new pectin-based material for selective low density lipoprotein-cholesterol removal has been reported (Lewinska *et al.*, 1994). The physiological effect of low-molecular-weight pectin is discussed by Yamaguchi *et al.* (1994). Such pectins that can retain their activities are important since high viscosity reduces their usability. This preparation exhibited high solubility and a repressive effect on lipid accumulation in the liver (Yamaguchi *et al.*, 1994). Pectin formulations have also been used for colonic drug delivery (Ashford *et al.*, 1994).

High-methoxy pectin loses about 5% of its USA-SAG grading when stored at 20°C in a dry atmosphere, whereas low-ester pectin is more stable and under favorable conditions loss is undetectable (Food Chemicals Codex, 1972; Anon., 1978; Anon., 1981c). The microbiological purity of pectins is specified in many cases by the manufacturer, since it is used mainly in acidic media and, therefore, yeast and mold counts are relevant. Typical specifications may include a total plate count at 37°C of less than 500 cells g^{-1}; a yeast and mold count at 25°C of less than 10 cells g^{-1} and *Escherichia coli*, salmonella and staphylococcus test results being negative.

5.7 Solution properties

Good pectin solubility can be achieved by following recommended dissolution procedures. In general, pectin is not soluble under conditions in which it forms a gel. The powder needs to be dispersed in warm water (not less than 60°C), at reduced mixing rates and then at full speed. Ignoring manufacturer recommendations could result in the formation of lumps that are difficult to dissolve. Good dissolution is achieved by mixing pectin with five times its own weight of sugar. Other blending media, such as a 65% sugar solution or alcohol to wet the pectin for small-scale laboratory use, are recommended. If a high-shear mixer is not used, then boiling for 1 min is necessary to guarantee full dissolution (Rolin and De Vries, 1990).

5.7.1 Viscosity

The viscosity of pectin solutions is dependent on their concentration, presence of calcium or similar non-alkali metals, pH, the chemical properties of the pectin, the DE and the average molecular weight. Dilute pectin solutions (up to approximately 0.5%) are Newtonian and only slightly affected by calcium ions. Increased pH results in increased viscosity. Salts of monovalent cations reduce pectin solution viscosity, because of reductions

at high ionic strength. The higher the average molecular weight, the higher the solution viscosity. The molecular weight of pectin can be estimated by using intrinsic viscosity methods. Pseudoplastic solutions can be achieved with concentrations higher than 1%. In contrast to dilute solutions and in the absence of calcium, such solutions increase in viscosity if the pH is reduced within the typical application range of 2.5–5.5. Pectins in the presence of calcium form thixotropic solutions, the viscosity of which increases with increasing pH within the aforementioned range. In fact, different textures can easily be achieved by combining pectin types and concentrations, ion concentrations and pH (Michel *et al.*, 1982; Christensen, 1954; Berth *et al.*, 1982). Solution properties of pectins are changed by hydrolysis of side-chains. Hydrolysis did not affect the specific viscosity of dilute (0.5%) pectin solutions; however, viscosity significantly decreased in concentrated 2.0–6.0% pectin solutions. Results suggest that pectin side-chains exist in an entangled state in concentrated solutions. In these latter solutions, the extent of viscosity reduction was dependent on pectin concentration (Hwang and Kokini, 1995). Based on viscometry measurements, the average molecular weight of commercial pectin normally falls between 50×10^3 and 150×10^3. It is important to note that by using other techniques such as light-scattering, other results ($\sim 1 \times 10^6$ or higher) have been found owing to intermolecular associations and aggregation of pectin molecules.

5.7.2 Chemistry and properties

Pectin is in fact a polyacid. The negative charge on dissolved pectin is smaller at low pH than at high pH. This charge attracts protons and, therefore, dissociations of individual acid groups are not independent (Rinaudo, 1974). Via a mechanism known as 'membraneless osmosis', pectin can concentrate solutions of proteins such as milk proteins. Since pectin and the protein cannot exist in the same solution, two phases develop, one rich in pectin and the other in protein. Pectin has a higher affinity for water and the protein phase is thus concentrated by a factor of 5–12. The addition of metal ions to pectin solutions causes an increase in viscosity, or gel formation or pectin precipitation. Reactions of polyanions (pectin) with polycations (other macromolecules) form insoluble products. Dissolved pectin exhibits good stability at pH 4. Far from this optimum, depolymerization occurs at low pH, whereas at high pH (any pH > 5) degradation occurs owing to β-elimination. High-ester pectins are more vulnerable to such degradation than their low-ester counterparts. In the juice industry, pectin-degrading enzymes are often used to obtain a clarified product.

A study of synergistic interactions in dilute polysaccharide solutions was recently performed (Goycoolea *et al.*, 1995). A simple viscometric approach was used in cases in which exclusion effects should be negligible. There were

no viscosity changes for alginate and pectin with sufficient calcium ions to induce almost complete conversion to the dimeric 'egg box' form, demonstrating that conformational rigidity is not, in and of itself, sufficient for other polysaccharides to form heterotypic junctions with mannan or glucomannan chains.

Atomic force microscopy (AFM) was used for imaging polysaccharides such as pectin, ι-carrageenan, xanthan and acetan (Kirby *et al.*, 1996). The polysaccharides were deposited from an aqueous solution onto the surface of freshly cleaved mica, air-dried and then imaged under alcohols. Improved resolution was obtained relative to the more traditional metal-coated samples or replicas (Kirby *et al.*, 1996).

5.8 Pectin gels

High-ester pectin gels can be successfully prepared following good dissolution. Jam preparation procedures can be found elsewhere. Briefly, they include heating the sugar and fruit fraction in amounts that will yield 65% soluble solids in the final batch. The pectin is added in solution form and stirring and boiling are carried out under vacuum to achieve the desired soluble-solids content. The vacuum needs to be broken before heating to pasteurization. Then citric acid is added to reduce the pH to 3.0–3.1. The mixture is cooled to filling temperatures and gelation occurs in the container itself. For high-ester pectin gelation, low pH, a high soluble-solids concentration and appropriate temperatures are needed to fulfill the desired requests. A high-ester pectin gel cannot be melted after solidification. Pregelation phenomena (stirring while gelation is in progress) result in lower-strength gels, or the absence of gelation with continued interference. For gel formation, a three-dimensional network is necessary to hold water, sugar and other solutes (Fig. 5.5). The junction zones in the high-ester pectin gel network have been described by a model suggested by Walkinshaw and Arnott (1981b). According to this model, three to ten polymer-chain segments with a helical structure form aggregates of parallel chains that are limited in size because of steric barriers, entropic factors and possibly rhamnose insertions (Christensen, 1986). Local crystallization is sustained by intermolecular hydrogen bonds and is probably reinforced by hydrogen bonding with water molecules in one set of triangular channels, and hydrophobic attractions between methyl groups forming columns in a second set of triangular channels. In molecular gel networks, at least two types of bonding are involved. One is strong and responsible for the elastic properties of the gel and the other is weaker and capable of reforming after disruption. Sugars play an active role in the formation of the pectin gel network by associating with pectin molecules via hydrogen bonding to form secondary links that reinforce the molecular network structure. The aging

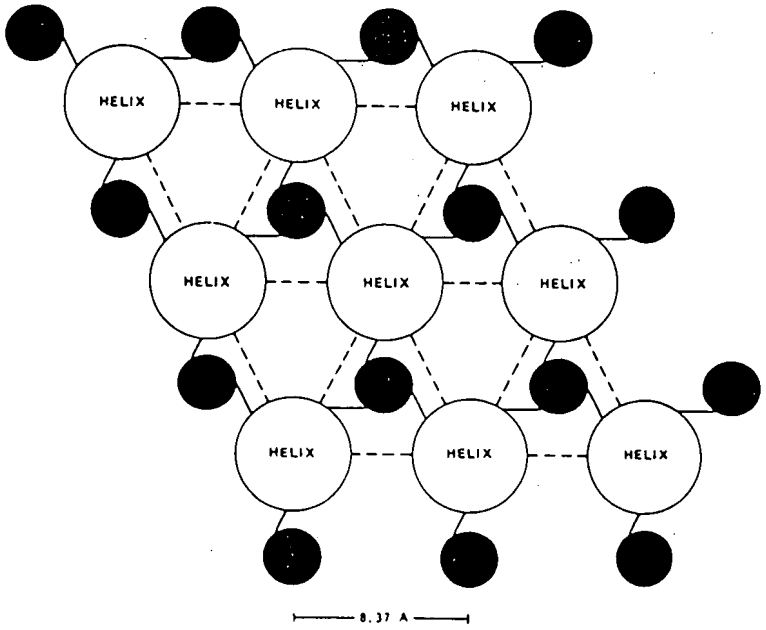

Figure 5.5 Junction zone in a high-ester pectin gel. (From Walkinshaw and Arnott, 1981b.)

process of high-methoxy pectin (HMP)–sucrose aqueous gels can be followed by low amplitude oscillation (Dasilva and Goncalves, 1994). Dynamic mechanical measurements enabled a determination of the point at which the system undergoes the sol–gel transition. The HMP–sucrose system is extremely sensitive to temperature variations during aging, especially in the lower temperature range. The gel's viscoelastic behavior indicates changes with aging temperature, probably because of variations in the mobility of the pectin chains and, consequently, in the lifetime of the junction zones (Dasilva and Goncalves, 1994). HMP–sugar gels are formed by a combination of hydrogen bonding and hydrophobic effects. Because magnitudes of the latter are affected by the solute used and temperature, gel strength and rate of structure development are also affected. Technologically important events that take place during the sol–gel transition have been considered, including a profile of complex viscosity during gelation, and the effects of rate of cooling and pectin concentration (Dasilva and Rao, 1995). Structure developments in HMP–fructose gels were also characterized by Rao and Cooley (1993, 1994). Weaker pectin networks are formed under thermal conditions unfavorable to the development of hydrophobic interactions. Gelling time and elastic modulus have a complex dependence on temperature, which can be attributed to the different thermal behaviors of the intermolecular interactions that stabilize the non-permanent cross-links of

these physical networks. The influence of temperature on the dynamic and steady-shear rheology of pectin dispersions was studied by Dasilva *et al.* (1994). The authors used the time–temperature superposition principle to calculate activation energies, and their dependence on temperature and shear rate was analysed.

Low-ester pectin gels do not require a high solids content or low pH, but they do need the presence of calcium, which can be provided by the fruit pulp if a fruit product is desired. Calcium binding to low-ester pectin cannot be explained as a simple electrostatic interaction: it involves intermolecular chelate binding of the cation leading to the formation of macromolecular aggregates (Kohn and Luknar, 1977). An 'egg-box' model has been suggested for primary junction zones in the low-ester pectin molecular gel network (Rees, 1982). Chain segments with 14 or more residues having a ribbon-like symmetry are believed to form parallel-oriented aggregates. Chelate bonds with oxygen atoms from both galacturonan chains formed by calcium ions are formed when calcium ions fit into 'cavities' in the structure (Fig. 5.6). Although they differ from those involving high-ester gels, the concepts of good manufacturing practice need to be maintained, and the ingredients appropriately selected. Main differences between high- and low-ester systems are the ability to melt a low-ester pectin gel and the immediacy with which solidification occurs in the low-ester pectin system, relative to the slow rate of the high-ester pectin gel. Amidated low-ester pectins are usually able to jellify preserves, jams and jellies with calcium ions originating from fruit and water (Broomfield, 1988). Non-amidated low-ester pectins generally require a higher calcium level and the addition of extra calcium is very often necessary to obtain proper gel formation (Christensen, 1986). The degree of amidation and esterification controls the readiness of low-ester pectin reactions with calcium to induce gel formation. Low-ester pectins with a DE of 25–35% (non-amidated), and pectins with 20–30% DE and 18–25% DA are highly reactive with calcium and are, therefore, used in low-calcium and low-soluble-solids content systems. Pectins with a low ester content of 35 to 45% (non-amidated) and those with 30–40% DE and

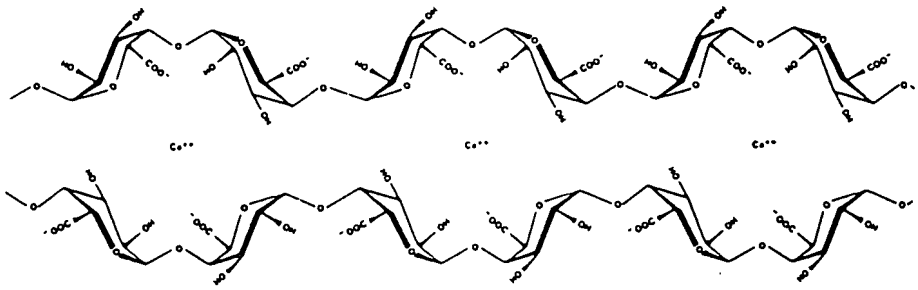

Figure 5.6 Junction zone in a low-ester pectin gel.

10–18% DA, because of their lower calcium reactivity, can serve in high-calcium or high-soluble-solids content systems (Buhl, 1990).

5.8.1 Gel properties

Results of gel-strength measurements yielded by different methodologies are not necessarily correlated. This is partly the result of the different properties defined and of the different measures such as deformation within the elastic limits and the force needed to break the gel. Traditional methods are the SAG method (this traditional method, based on Cox and Higby's work (1944) and adopted by the Institute of Food Technologists (1959), defines a jelly grade designation of 150° USA-SAG as the transformation of 150 parts sucrose by 1 part pectin into a gel of 65° Brix, pH 2.2–2.4 and a gel strength of 23.5% SAG after being cast for 2 min and removed from a standard glass with exactly specified inner dimensions), the use of the LFRA texture analyser, the Boucher Electronic Jelly Tester, the FIRA tester, the family of universal testing machines, the Herbstreith pectinometer, a spreadameter and the Bostwick Consistometer. In addition to the variety and plurality of gel texture measurements, other measurements of time–temperature relationships have also been developed within the industry. Gelation is influenced by the degree of methyl esterification, amidation, pectin concentration, water activity, the presence of calcium ions and pH.

The profile of shear modulus temperature meltdown of pectin gels was described by Clark *et al.* (1994). In this study, a cascade-theory approach to biopolymer gelation was developed to describe variations in the shear modulus with temperature for thermoreversible gels. The broadness of this 'melting transition' is seen to depend critically on the enthalpy of cross-linking, whereas the critical gel-melting temperature is determined by additional factors, such as the entropy of cross-linking, polymer concentration, molecular weight and the number of cross-linking sites. When the model was used to fit experimental data from a pectin system, a broad melting transition and the high melting point of the pectin system were consistent with much smaller negative values for these parameters (Clark *et al.*, 1994).

The mechanisms of gelation hint at a combination of hydrogen bonding and hydrophobic interactions in the case of high-ester pectin gel formation. The hydrophobic parts of the high-ester pectin molecule are the ester groups. Contact between these hydrophobic areas is associated with energy contribution. Hydrogen bonds formed between adjacent galacturonan chains contribute even more to decreases in the energy of junction-zone formation. However, the energy contribution of the hydrophobic interaction is necessary in order to make the sum of the energy contributions favoring gelation large enough to exceed energy contributions that resist gelation. The proposed mechanism suggests that gel formation with high-ester pectins

relates to the rigidity of its molecule, its correlation to DE level and the presence of sugars in the system. For low-ester pectin, the 'egg-box' model used to explain alginate gelation is proposed. Twofold helices are bridged by calcium ions of opposing carboxyl groups. In dried pectins, helices with three subunits per turn are detected, hinting that helix structure changes from twofold to threefold when the gel is dried to a powder. Support for the 'egg-box' model also comes from the direction of equilibrium dialysis: ~50% of the calcium ions cannot be removed by exposure to a very large concentration of univalent cations.

5.9 Applications

The most common use of pectins is in the preparation of jams, jellies, or similar gels. Detailed information on jam production can be found elsewhere (Kertesz, 1951). Ordinary jam is generally made from high-ester pectin, whereas the low-ester pectins are used when a softer, more spreadable texture is desired. If fruit particles (pulp) are to be contained in the jam, a high gelation temperature is used and solidification begins almost immediately after filling the containers, with almost no floatation of the particles being observed. If very large containers are used for jam-filling, then pectins with lower filling temperatures should be considered, to minimize flavor and color destruction, especially in the center of the container. The rheological indices of fruit content in jams and the effect of formulation on flow plasticity of sheared strawberry and peach jams has been studied (Costel *et al.*, 1993). The effect of formulation factors on Casson yield values measured at low and medium shear rates are reported.

To prepare low-sugar (less sweet) gums, low-ester pectins are used in combination with calcium in an amount related to gelation temperature and the quality of the formed texture. When jellies that contain no particles are produced, slow-setting pectins that solidify a long time after filling, allowing air bubbles to float and escape from the product, are preferred. For confections, a slow-setting high-ester pectin is used. The solid content of such preparations is high, ~78%, in contrast to ordinary jams at ~65% or low-sugar jams at ~30–55% (Rolin and De Vries, 1990). For baked goods, a heat-resistant gel is usually produced with a soluble-solids content of 45–75% and, depending on the type of pectin used, a typical dosage yields pH values of 3.3–3.6; if a cold-setting gel is used, a product with ~61% soluble solids and pH 4.0 is produced using rapid-set high-methyl-ester pectin (0.7%). Heat-resistant gels are generally prepared from high-ester pectins but can be produced from low-ester pectins if calcium citrate is used in the formulation to elevate the gelation temperature subsequent to the setting of the system. Fruit preparations for dairy products are often sold as semi-gel/thixotropic products with a typical soluble-solids content of 30–

65% and a pH of 3.6–4.0, usually prepared with 0.3–0.6% low-methyl-ester pectin. These should be prepared in such a way that the big fruit chunks or berries are distributed uniformly even after storage and pumping or transport (Rolin and De Vries, 1990).

Pectins are used to prepare bakery fillings and glazes. Oven-resistant high-sugar jams are produced at a solids content of ~70% using rapid-set pectin. Another demand of such products is mechanical stability. The less the gel is ruptured, the lower the syneresis at elevated heating temperatures. Non-amidated low-ester pectins are recommended for the production of bakery jams with satisfactory stability. Low-ester pectin gels are produced with ~65% soluble solids and a relatively high dosage of calcium-reactive, low-ester pectin. Before being applied to the baked goods, water is added, then the gel is heated to ~85°C to induce melting, and hot coating of the product follows. Upon cooling, a glossy coverage is formed. Other studies on the physical and mechanical properties of highly plasticized pectin–starch films can be found (Coffin and Fishman, 1994).

The thermomechanical properties of pectin and polyvinyl alcohol (PVA) blends have been recently studied (Coffin *et al.*, 1996). Increasing the amount of PVA in the blends reduced the storage and loss modulus of the films above the glass transition temperature. Changes in the molecular weight and degree of ester hydrolysis of PVA exerted a rather small effect on the blends. The composition should be targeted to the specific aim (Coffin *et al.*, 1996).

Stabilization of pasteurized or sterilized, acidified milk products (pH values of ~3.5–4.2) can be achieved by using high-ester pectins with DE greater than ~70. Acidification can be produced by either fermentation or the addition of fruit juice. If casein stabilization is not achieved, an undesirable grain-like texture is obtained. The pectin, added before homogenization, is absorbed onto the casein particles, which have a positive charge in the unstabilized milk. If the amount of added pectin is small, then the charge is neutralized and the system tends to collapse owing to the removal of repulsive forces. If pectin addition is continued however, a new repulsive force builds up, resulting in stabilization of the acidified milk system. Hydrophobic as well as electrostatic interactions are important in stabilizing pectin–casein dispersions (Pereyra *et al.*, 1995). The shear rate and time dependency of stirred yoghurt rheology were evaluated as influenced by added pectin and strawberry concentrate (Basak and Ramaswamy, 1994). The rheology of the flavored yoghurt was influenced by both pectin (0–0.5%) and the concentrate, and the desired product viscosity could be obtained by postfermentation mixing of stirred yoghurt with the pectin and fruit concentrate. Quality requirements for yoghurt–fruit preparations and the rheological parameters used to assess their properties are discussed in Kratz and Dengler (1995). Three different types of pectin were compared in yoghurt preparations. The possibility of using yoghurt–fruit

preparations instead of increasing the dry matter content in order to improve the consistency of fruit yoghurt was considered. In fat-free yoghurt, good mouthfeel and stability were achieved by increasing the percentage of milk solids and adding a mixture of gelatin, starch and pectin (Moller, 1995). In order to eliminate unacceptable viscosity, poor mouthfeel and syneresis in pasteurized yoghurt, processing conditions need to be adjusted and starch, gelatin and pectin added (Moller, 1995). The use of non-traditional additives (dried fruit and vegetable powders) in the manufacture of cultured milk products for therapeutic and prophylactic uses was reported by Arkhipova and Krasnikova (1994). Results of clinical trials showed that in less than 2 weeks patients receiving these cultured milks shared increased appetites, improved intestinal microflora and 20–25% decreases in blood cholesterol level (Arkhipova and Krasnikova, 1994).

Pectin is also used to stabilize clouding in beverages. Such stability is dependent on the nature and amount of the pectin present. Natural clouding agents can be produced (Elshamei and Elzoghbi, 1994) from orange and lemon peels using enzyme preparations to hydrolyse the pectin in the peel. The chemical and physical properties of the clouds were evaluated in parallel to the drink's properties, taste and stability. The cloudiness of the produced drinks stabilized after 42 days of storage at 25°C. Other reports on the physicochemical nature of pectin associated with commercial orange juice clouding can be found elsewhere (Klavons *et al.*, 1994).

The confectionery industry makes use of slow-set high-ester pectins to prepare fruit jellies and jelly centers. Low-ester pectins are also used to impart thixotropic behavior at low concentrations, or to achieve a cold-set type of gelation if diffusion of calcium ions occurs. The use of pectin in confections permits the manufacture of products with tailor-made textural properties, good flavor release and compatibility with continuous processing. High-methoxy pectin was also reported to produce coatings that inhibit lipid migration in a confectionery product (Brake and Fennema, 1993).

Low-ester pectins are used as gelling agents and texturizers in many very different food products such as artificial caviar, meat products and dessert jellies. Pectin–alginate combinations have a synergistic effect in terms of gel-formation properties. Xanthan and pectin together can serve as an appropriate stabilizer for salad dressings. The incorporation of pectin in water-ice and sherbet preparations improves product acceptability by minimizing the growth of ice crystals. Galactomannans in combination with pectin serve to stabilize ice cream. Pectins are used to stabilize emulsions. Modified pectins in whey–protein emulsions (Einhornstoll *et al.*, 1996) were found to stabilize the whey protein at high enough concentrations. For any individual utilization, the most suitable pectin needs to be selected. Frozen fruit preparations are improved by incorporating pectin into the product. Pectins can be used for coating, in recipes of spray-dried instant tea and for many other products.

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Pectins are mixtures of polysaccharides that originate from plants, contain pectinic acids as major components, are water soluble, and are able to form gels under suitable conditions (See section on Physical Properties).

In this chapter, as is frequently done, the term pectin will be used in a generic sense to designate those water-soluble galacturonoglycans of varying methyl ester content and degree of neutralization that are capable of forming gels under suitable conditions (See section on Physical Properties), i.e., other polysaccharides that may be present in commercial mixtures will be ignored.

Pectins are subdivided according to their degree of esterification (DE), a designation of the percent of carboxyl groups esterified with methanol. Pectins with DE > 50% are high-methoxyl pectins (HM-pectins); those with DE < 50% are low-methoxyl pectins (LM-pectins).

The degree of amidation (DA) indicates the percent of carboxyl groups in the amide form (See section on Chemical Properties).

Structure

For a more detailed discussion of the chemical structure of pectins, see reference 3.

Pectin, a structural, cell-wall polysaccharide of all higher plants, like most other polysaccharides, is both polymolecular and polydisperse, i.e., it is heterogeneous with respect to both chemical structure and molecular weight (4). From molecule to molecule, in any sample of pectin, both the number and percentage of individual monomeric unit types will vary, and the average composition and distribution of molecular weights can vary with the source, the conditions used for isolation, and any subsequent treatments. Because both parameters determine physical properties, various functional types of pectin can be produced by controlling the source, isolation procedure, and subsequent treatment(s)

Pectin is primarily a polymer of D-galacturonic acid. The principal and key feature of all pectin molecules is a linear chain of (1→4)-linked α-D-galactopyranosyluronic acid units, making it an α-D-galacturonan [a poly(α-D-galactopyranosyluronic acid) or an α-D-galacturonoglycan].

In all natural pectins, some of the carboxyl groups are in the methyl ester form. Depending on the isolation conditions, the remaining free carboxylic acid groups may be partly or fully neutralized, i.e., partly or fully present as sodium, potassium or ammonium carboxylate groups. The ratio of esterified D-galacturonic acid units to total D-galacturonic acid units is called the degree of esterification (DE) and strongly influences the solubility, gel forming ability, conditions required for gelation, gelling temperature, and gel properties of the preparation.

In pectin from some sources, some of the units occur as O-2 or O-3 acetates. Such esterification hampers gelation, so much so that complete inhibition of gelation occurs when one out of eight

of counterions in the solution. For example, addition of monovalent cations effects a reduction in viscosity, the degree of which is greater with decreasing DE. Addition of salts of di- and trivalent cations has an opposite effect (20). In general, viscosity, solubility, and gelation are related, i.e., factors that increase gel strength, for example, will increase the tendency to gel, decrease solubility, and increase viscosity, and vice versa.

These physical properties of pectins are a function of their structure which is that of a linear polyanion (polycarboxylate). As such, monovalent cation salts of pectins are highly ionized in solution, and the distribution of ionic charges along the molecule tends to keep it in an extended form by reason of coulombic repulsion (21). Furthermore, this same columbic repulsion between the carboxylate anions prevents aggregation of the polymer chains. (The number of negative charges is, of course, determined by the DE.) In addition, each polysaccharide chain, and especially each carboxylate group, will be highly hydrated. Solutions of monovalent salts of pectins exhibit stable viscosity because each polymer chain is hydrated, extended, and independent.

Because the commercial importance of pectin is predominately the result of its unique ability to form spreadable gels in the presence of a dehydrating agent (sugar) at a pH at or near 3 or in the presence of calcium ion (jams, jellies, and marmalades made from fruit juices or whole fruit), that is the property most often studied and focused upon. Factors that determine whether gelation can occur and that influence gel characteristics are pH, concentration of cosolutes (sugars), concentration and type of cations, temperature, and pectin concentration. The ways in which these factors influence gelation are dependent upon the following molecular properties of the specific pectin: molecular weight (4), degree of esterification (DE), degree of amidation (DA), presence of acetate esters, and heterogeneity. All these parameters are interdependent. In general, under similar conditions, the degree of gelation, the gelling temperature, and the gel strength are generally proportional to each other and each property is generally proportional to the molecular weight and inversely proportional to the DE.

As the pH is lowered, i.e., as the hydrogen ion concentration of the solution is increased, ionization of the carboxylate groups is repressed, i.e., the highly hydrated carboxylate groups are converted into only slightly hydrated carboxylic acid groups. As a result of losing some of their charge, the polysaccharide molecules no longer repel each other over their entire length; and as a result of losing some of the water of hydration, they can associate over a portion of their length to form a gel. Apparent pK values (pH at 50% dissociation) vary with the DE of the pectin (22); a 65% DE pectin has an apparent pK of 3.55, while a 0% DE pectic acid has an apparent pK of 4.10. However, pectins with increasingly greater degrees of methylation will gel at somewhat higher pH, undoubtedly because they have fewer carboxylate anions at any given pH (See later paragraph). pH affects gel texture more than gel strength.

Exhibit C

Table I. Pectin and Unavailable Carbohydrate Content of Fruits, Nuts, and Vegetables

	Dry matter	Pectin		Unavailable carbohydrate
		Ca Pectate	Carbazol	
	Fresh weight of edible portion (%)			
Fruit				
Apple	15.9	0.78	0.45	1.7
Apricot	13.4	1.00	0.70	2.1
Banana	29.3	0.94		3.4
Blackberry	18.0	0.94	0.30	7.3
Blueberry	16.8		0.30	
Cherry	18.5	0.39	0.36	1.7
Fig	15.4	1.11		2.5
Grape	19.3	0.19	0.20	0.4
Grapefruit	9.3	3.90		0.6
Lemon	14.8	2.90		5.2
Loganberry	15.0	0.59		6.2
Orange	13.9	2.36		2.0
Peach	13.8	0.39	0.64	1.4
Pear	17.0	0.49	0.46	2.5
Pineapple	15.7	0.09		1.2
Plum	15.9	0.44	0.59	2.1
Raspberry	16.8	0.97	0.34	7.4
Rhubarb	5.8	0.44	0.34	2.6
Strawberry	11.1	0.75	0.50	2.2
Watermelon	7.4	0.18		
Nuts				
Peanut	95.5	5.98		8.1
Walnut	76.5	5.80		5.2
Vegetable				
Asparagus	7.6		0.22	1.5
Avocado	7.8	2.86		
Bean	8.4	0.70	0.55	3.0
Beet	12.9	0.91	0.42	3.1
Broccoli	9.2		0.49	4.2
Brussels Sprout	9.2		0.78	4.8
Cabbage, red	10.3		0.53	3.4
Carrot	10.2	2.00	0.96	2.9
Cauliflower	10.9		0.38	1.5
Cucumber	3.6	0.16	0.17	0.4
Eggplant	6.6	0.47		2.5
Garlic	38.7	1.11		
Kohlrabi	9.7		0.38	
Lettuce	4.8		0.34	1.4
Okra	11.1	1.53		
Onion	7.2	0.35	0.44	1.3
Pea	21.5		0.34	5.2
Pea, with pod		0.57		
Pepper, green	16.6	0.09		
Potato	24.2	0.83	0.34	2.1
Pumpkin	5.3	1.24	0.20	0.5
Radish	6.7		0.45	1.0
Rutabaga	13.0		0.80	
Soybean	90.0	3.45		
Spinach	14.9		0.33	6.3
Sweet potato	28.0	0.78		2.1
Tomato	6.6	0.20	0.30	1.5
Turnip	6.7	0.29		2.8
Yam	26.5	0.62		

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Exhibit D

pectic acid, and pectinic acid all occur in the solid state (fibers) as right-handed (3_1) helices with a three-fold screw axis (trisaccharide repeat) (12-15). In solid pectinic acid, the polymer molecules pack so that the chains are parallel to each other; the pectates pack as corrugated sheets of antiparallel chains (14,15).

It is further suggested that junction zones in pectinic acid (HM-pectin plus sucrose) gels are formed by a columnar stacking of methyl ester groups to form cylindrical hydrophobic areas parallel to the helix axes. Two models for the formation of junction zones in calcium pectate (LM-pectin) gels have been proposed. One suggests an aggregation of chains by a crosslinking of carboxylate anions with calcium ions to form a structure similar to that of the corrugated sheets of antiparallel helices (3-6 chains in an average junction zone) found in solid calcium pectate (15). The other is the "egg box" model used to describe the formation of calcium alginate gels (16,17). This model is proposed because of the close similarity between (1→4)-linked poly(α-D-galacto-pyranosyluronic acid) segments of pectic acid and (1→4)-linked poly(α-L-gulo-pyranosyluronic acid) segments of alginic acids, segments that are mirror images except for the configuration at C-3. From circular dichroism and equilibrium dialysis studies, it has been concluded that interchain association of hydrated pectinic acid molecules, in the presence of swamping levels of monovalent counterions, is limited to the formation of dimers of chains of 2_1 helical symmetry with specific site-binding of calcium ions along one face of each participating chain (18,19; see also reference 20). When Ca^{2+} is the sole or principal counterion, these dimers further aggregate without rearrangement, leading to an approximate doubling of the amount of Ca^{2+} bound cooperatively (18,19). Based on available information, the Unilever Research group (19) has concluded that drying of a calcium pectinate gel effects a polymorphic phase transition in which associated, regular, buckled chains with two-fold symmetry ("egg box") as found in L-guluronoglycan chain segments are converted into associated chains with three-fold symmetry as found in solid state calcium pectinate (12-15). It should be noted that the axial-axial linkages in a chain of aldohexopyranosyl units linked 1→4 gives a buckled conformation naturally (Figure 1) and that the gel structure(s) is(are) as yet not well understood.

Physical Properties

Pectins are soluble in pure water, but they are insoluble in aqueous solutions in which they would gel at the same temperature if dissolved at a higher temperature. Monovalent cation (alkali metal) salts of pectinic and pectic acids are usually soluble in water; di- and trivalent cation salts are weakly soluble or insoluble.

Although pectins are not employed as thickening agents, pectin solutions exhibit the non-Newtonian, pseudoplastic behavior characteristic of most polysaccharides. As with solubility, the viscosity of a pectin solution is related to the molecular weight, DE, and concentration of the preparation and the pH and presence

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Amidation results in a higher gelling temperature and a decreased need for a divalent cation.

The distribution of carboxyl/carboxylate groups also affects gelation. Pectins with blocks of methyl ester and carboxyl groups (as opposed to a random distribution) generally produce weaker gels and have a greater requirement for divalent cations.

Chemical Properties

Dissolved pectins undergo deesterification and depolymerization in aqueous systems. The pH of greatest stability is about 4. At pH values both above and below 4, deesterification and depolymerization occur concurrently, with the rate of deesterification being greater than the rate of depolymerization. The presence of solutes, which lowers water activity, reduces the rates of both reactions.

Deesterification occurs by normal acid- and base-catalyzed mechanisms of ester hydrolysis. Depolymerization at low pH values occurs by means of acid-catalyzed hydrolysis of glycosidic bonds (24). Acid-catalyzed hydrolysis occurs preferentially at the L-rhamnopyranosyl glycosidic bonds. Hydrolysis of these linkages produces galacturonoglycan chains with a degree of polymerization of about 25 (6,9). Side chains, particularly those containing L-arabinofuranosyl units, should also be preferentially removed by hydrolysis because of the inherent stability to acid-catalyzed hydrolysis of glycuronosyl glycosidic bonds and the inherent lability of furanosyl glycosidic bonds (24). However, if the side chains are attached to rhamnogalacturonan sequences (8), it should not be possible to convert "hairy" regions to "smooth" regions by treatment with acid because the lability of the L-rhamnopyranosyl bonds would result in concurrent depolymerization of the main chain.

At pH values of 5-6 pectin solutions are stable only at room temperature. As the temperature is raised, pectin chains cleave by a beta-elimination reaction (25-39) (Figure 1), a reaction which is stimulated by organic anions (40). Deesterification of pectin proceeds simultaneously with the beta-elimination depolymerization reaction, which occurs only at monosaccharide units that are esterified. At pH values above 6, deesterification and depolymerization are rapid reactions even at room temperature, the rate of each reaction increasing with increasing pH.

Hydroxyl-group reactions, such as etherification, acetalation (41), esterification (42-51), and oxidation, can be done in the same manner as they are on other polysaccharides. Esterifications (47) of carboxyl groups and interactions with cations, including polycations such as proteins below their isoelectric pH, occur as they do with other glycuronoglycans. Reduction of carboxyl groups to hydroxymethyl groups has been done with diborane (52, see also 53) and by borohydride treatment of methyl and hydroxyethyl esters (54). Reduction of carboxyl groups which have been activated with a water-soluble carbodiimide should be straightforward (55,56).

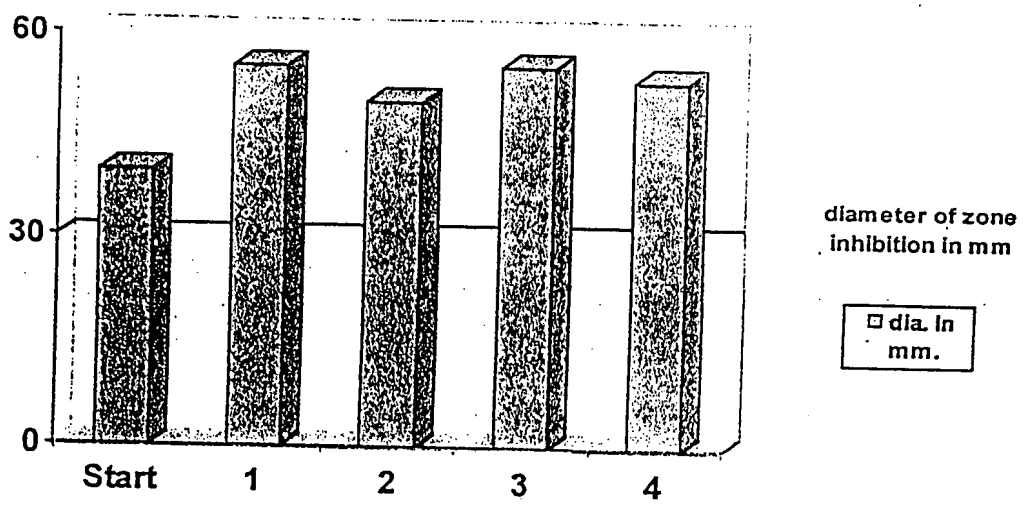
When ammonia (57-61) is used to prepare LM-pectin from HM-pectin, some of the methyl carboxylate groups are converted into carboxamide groups, producing "amidated pectin". The

TABLE 1

In-Vitro Reusability of Silver Alginate (**ALGIDEX AG**)

Materials & Methods:

A one (1) square inch piece of silver alginate dressing (ALGIDEX AG) was checked for reusability against *Ps. Aeruginosa.* Nutrient agar in which, the agar content was increased to 10% was inoculated with 0.1 ml of actively growing culture and surface spread. Hard agar was used to prevent too rapid water uptake from the gel. The Algidex Ag was placed on the agar surface and diffusion was allowed to commerce for 3 hours in refrigeration. The plate was then incubated at 37° Celsius for 16 hours. The zone of inhibition in mm was measured and the gel was then transferred to another inoculated agar plate. This process was repeated 4 times.



Silver-Coated Dressing Acticoat Caused Raised Liver Enzymes and Argyria-like Symptoms in Burn Patient

Marija Trop, MD, Michael Novak, MD, Siegfried Rodl, MD, Bengt Hellbom, MD, Wolfgang Kroell, MD, and Walter Goessler, PhD

Background: Treatment of acute burn wounds with silver sulfadiazine (SSD) has raised concern about potential silver toxicity. Numerous adverse reactions and side effects have been reported and an increasing resistance to SSD, especially in Pseudomonas strains, have motivated researchers to search for an alternative wound dressing.

Methods: Recently, a silver-coated wound dressing Acticoat (Smith & Nephew, Inc.) has become available for use in burn patients. It is a three-ply dressing, consisting of an inner rayon/polyester absorptive core between two layers of silver-coated, high-density polyethylene mesh. In a moist environment, the nanocrystals of silver are released and improve the microbial control in the wound.

Results: After 1 week of local treatment with Acticoat in a young, previously healthy 17-year-old boy with 30% mixed depth burns, hepatotoxicity and argyria-like symptoms, a grayish discoloration of the patient's face, appeared. The silver levels in plasma (107 μg/kg) and urine (28 μg/kg) were clearly elevated, as well as the liver enzymes. As soon as the local application of Acticoat was aborted, the clinical symptoms and liver enzymes returned to the normal values.

Conclusions: This is the first report on silver toxicity in a patient with 30% burns who received Acticoat for local treatment. Due to substantial experiences with adverse SSD reactions and side effects, it is appropriate to keep the possibility of a toxic silver effect in burn patients treated with Acticoat silver-coated wound dressing in mind. The silver levels in plasma and/or urine should be monitored.

Key Words: Burns, Acticoat, Silver toxicity.

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The destruction of a large area of the skin is a life-threatening condition due to the loss of essential body fluids and possible occurrence of wound infection, which may lead to septicemia. The core events in the management of large, deep burns are wound excision and closure.[1] In superficial burns and smaller mixed-depth thermal injuries, one usually has time to allow the wound to fully evolve to a point where it is easy to determine burn depth on physical examination. Therefore, safe coverage of the wound surface is necessary during that time. The aims of topical antimicrobial therapy have been to delay the growth of the patient's endogenous bacteria and to prevent the colonization of the dressed wound by external nosocomial organisms, while promoting the healing of the wound and minimizing the desiccation, pain and discomfort associated with dressing care.

The ideal skin substitute should be inexpensive; have a long shelf life; be usable off the shelf; be nonantigenic, durable, flexible, and prevent water loss; be a bacterial barrier, drape well, be easy to secure, grow with the child, and applicable in one operation; should not become hypertrophic; and—it does not exist yet.[2]

A variety of different factors have been proposed as having a negative impact on the speed of wound healing. One of the major contributors to delayed wound healing is a prolonged inflammatory response in the wound. Normally, the inflammatory response occurs following wounding and is initiated to help the body clean up tissue debris, counter any invading microorganisms, and signal the appearance of cells required for the synthesis of new tissue components. A prolonged inflammatory response may result in the destruction of tissue through the same mechanisms that normally have protective and restorative functions. The presence of bacterial cells within the wound is known to amplify and perpetuate the local inflammatory response. However, the presence of a high level of bacterial cells causes the prolonged inflammatory response in the absence of appropriate clinical measures to reduce the bacterial burden in the wound.[3]

Silver compounds are widely used as an effective antimicrobial agent to combat pathogens (bacteria, viruses, and eukaryotic microorganisms) in the clinic and for public health hygiene. Silver ions (Ag^+) are microcidal at low concentrations and have been used for decades as a topical antimicrobial agent for burns treatment. Elemental silver requires ionization for antimicrobial efficacy. It has a broad range of antimicrobial actions, including the disruption of the bacterial cell wall structure, the disruption of key bacterial enzymes such as cytochrome b and a_3, and interaction with the nucleic

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Exhibit H

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acids caused by its preferential binding to nitrogenous groups in guaninen and other nucleotides.[4]

CASE REPORT

While at work, a highly flammable solvent drenched the trousers of a 17-year-old apprentice boy. When he pulled a cigarette lighter out of his pocket, his trousers caught fire. He undressed immediately and cooled the wounds in the shower. The previously healthy young man sustained 30% total burn surface area (TBSA) superficial and deep dermal flame burns to his legs, buttocks, and hands. After initial resuscitation and analgesia at the local hospital, the patient was transferred to our burns unit 2 hours postinjury. On admission, he was in a stable clinical condition and on a Ringer-Lactate drip. He was 170 cm tall and weighed 61 kg. In the anamnesis, it was stated that he was allergic to wasp and bee venom, smoked moderately, and occasionally drank alcohol.

On admission, the patient was brought to the operating room for further wound evaluation and treatment. His body temperature was 36.3°C; he was conscious and pain free. The initial assessment of the buttocks and hands was that of partial thickness burns, and the leg burns were assessed to be deeper dermal burns that might require grafting. In anesthesia, the patient was washed completely, his wounds cleansed, and any loose or devitalized tissue were removed and rinsed with sterile water. The Acticoat (Smith and Nephew Inc.) wound-dressing was moistened with sterile water and applied to the complete burn area, followed by wet and dry sterile gauze dressing and secured in place with elastic netting. Blood samples were taken. Later on, the patient's body temperature was well maintained; he felt comfortable and was in a good mood. With additional intravenous fluid therapy, his vital parameters were stable and diuresis was adequate. Soon he regained his appetite and intravenous resuscitation was finished 24 hours later.

The Acticoat dressing was kept moist with distilled water and changed on day 4 postinjury. The wounds were checked, swabs taken, and, after cleansing, the Acticoat was reapplied. Macroscopically there were no symptoms of any local infection, no substantial exudate was present, but some clear appearing gel covered the wound in a few areas. The patient did not show any symptoms of septicemia and was doing well.

On day 6 postinjury, Acticoat was changed for the second time and the wound showed good healing progress without local infection.

At the same time, the patient gradually developed a grayish discoloration of his face with remarkable pale-bluish lips, but was not cyanotic. He appeared ill and complained about being tired and having no appetite; simultaneously he was found to have elevated aspartate aminotransferase, alanine aminotransferase, and gamma-galactosyl transferase liver enzymes. Total bilirubin, lactate dehydrogenase, and cholinesterase remained within the normal range. With the exception of low-dose paracetamol, the patient had not received any potentially hepatotoxic drugs. Serum methemoglobinemia was negative as well.

An abdominal ultrasound showed an only slightly enlarged liver and spleen with no focal abnormalities; the intestine, pancreas, gallbladder, and urogenital system were also without pathologic findings. His renal function was always normal.

Screenings for hepatitis A, B, and C viruses, as well as cytomegalovirus and Epstein-Barr virus, were negative. Wound culture swabs remained sterile or showed a growth of fecal strains in the inguinal burn wound (*Escherichia coli, Enterococcus* sp.) and moderate *S. aures* colonization on one knee.

The patient's general condition, laboratory results, and skin discoloration did not improve. On day 7 postburn, the blood and urine silver levels were checked and found to be profoundly elevated. Except Acticoat, no other source of silver was used on or by this patient. The Acticoat dressing was removed the same day and not used again. The local wound treatment was changed to a betadine (BI) ointment dressing.

In the following days, the dressings were changed at regular intervals. Initially with the Acticoat treatment, the wound had shown good healing progress, but after the local therapy regime had been changed, the healing progress had been disrupted. Simultaneously, the discoloration of the face gradually faded over a period of days and the liver function tests were returning to their normal values. On day 17 postinjury, when his clinical condition had changed for the better, 8% of the TBSA was excised and grafted. The take rate was satisfactory.

Finally, the patient made a full recovery and was discharged with still markedly elevated blood silver levels, 7 weeks postinjury. Ten months later silver was hardly detectable, neither in blood (0.9 μg/kg), nor in urine (0.4 μg/kg). Table 1 shows laboratory checks and local wound treatment.

DISCUSSION

There is much published evidence to show that silver is a potent biocide and the idea of including silver ions in dressings to combat or prevent local infection is attractive.

A 0.5% silver nitrate solution is the lowest concentration that remains active against bacteria in vitro and in vivo in burns and has no toxic effect on growing epidermal cells. $AgNO_3$ is active against *S. aureus*, hemolytic streptococci, and generally against *Pseudomonas aerugenosa* and *E. coli*. The main complication occurring during the treatment is a drop in serum sodium and chloride, due to precipitation between Na^+, Ag^+, and Cl^-, HCO_3^-, CO_3^- and protein anions, which leads to the production of very slightly soluble or insoluble salt solutions. In general, it was assumed that silver is not taken up by the body, but in postmortem examinations elevated silver levels in the kidney, spleen, liver, and muscles have been found.[5]

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Table 1 Laboratory Checks and Local Wound Treatment

	Reference Range	Day														
		1	4	7	8	9	10	11	12	15	16	17	22	27	49	97
WBC (5,550–14,000)/μL		13.2	8.3	10.1	12.1		13.7	13.7	12.2		9.6			6		
Hemoglobin (11.5–13.5) (g/dL)		15.7	14	13.4	14.1		13.6	13.5	13.4		13.7			12.1		
Platelet count (140,000–440,000)/μL		162,000	188,000	304,000	353,000		428,000	448,000	433,000		437,000			299,000		
Blood urea (10–45) (mg/dL)			15	26	22	18		22	21	20		18			23		
Serum creatinine (0.50–1.30) (mg/dL)			0.9	1	0.9	0.8		0.8	0.8	0.9		0.8			0.7		
Aspartate aminotransferase (−21) (U/l)			10	42	78	64		39	39	26		28			13	9	
Alanine aminotransferase (−22) (U/l)			8	69	233	193		143	134	111		90			45	11	
Gamma-galactosyl transferase (−19) (U/l)			8	24	94	96		78	71	63		48			29	14	
C-reactive protein (−8)(mg/L)			37	128	93	66		27	16	14		5			5		
Silver in blood (<0.21 μg/kg)*					107		100	104	104	99	85		83		71	42	13.3
Silver in urine (<0.21 μg/kg)*					28		15.1	13.6	10.3	6.8	9.8		4.5		3.8	2.3	1.5
Local treatment		Acticoat	Acticoat	Acticoat		BI	BI	BI	BI	BI	BI	BI	Surgury				

*Silver concentrations were determined with an Agilent 7500c inductively coupled plasma mass spectrometer (Agilent, Waldbronn, Germany) after microwave assisted mineralization of the serum and urine samples with nitric acid in an UltraCLAVE2 (EMLS, Leutkirch, Germany) at 250°C for 40 minutes.

The best known and the most widely used product is a topical cream that contains 1% silver sulfadiazine plus 0.2% chlorhexydine digluconate in a water immiscible cream base. The *Pseudomonas* strains nearly disappeared under the influence of silver sulfadiazine and the mortality in burn patients declined. Due to the absorption of silver from large bums and the possibility of silver toxicity occurring in some patients, the use of SSD came into question.[6–8]

Boosalis et al.[9] monitored plasma silver levels and urinary silver excretion in 23 patients with second- and third-degree burns who were treated with SSD. Mean serum concentrations were modestly elevated. In contrast to the serum levels, a high urinary excretion of silver was found, predominantly in patients with burns involving >60% TBSA, with peaks of 1,100 μg/24 hour (normal < 11 μg/24 hour). Coombs et al.[10] showed in their prospective study that plasma silver levels rapidly increased in bum patients treated with SSD. Twenty-two patients were studied; the age range was 17 to 80 years. Serum silver levels rose in all patients with burns >5% TBSA. The altered hepatic and renal laboratory results were not correlated with plasma silver levels.

SSD and silver, as an ingredient of the cream, have been linked to a variety of side effects and adverse reactions. The systemic effects range from allergic reactions[11] and erythema multiforme,[12] to the deterioration of the mental status[13] and frequent leukopenia. The drop in white blood cells (WBCs) occurs early in the course of the treatment and is not related to septic episodes. Withdrawal of SSD leads to a prompt recovery of the WBCs, but continuation of the SSD therapy does allow a slow WBC recovery as well. There is no increase in mortality with transient leukopenia.[14]

In addition to the reports on transient leucopenia[15–17] Gamelli et al.[18] showed in an in vitro study that SSD was directly cytotoxic to myelopoietic tissue and, in vivo, altered the myeloid cell compartment.

Due to a number of papers reporting side effects and adverse reactions to SSD, the high frequency of dressing changes and the fact that resistance to SSD, especially in *Pseudomonas* strains,[5] had been observed to arise, there was a demand for an alternative wound dressing for local burn wound treatment.

Recently, a silver-coated wound dressing, Acticoat has become available for use in burn patients. It contains a three ply-dressing, consisting of an inner rayon/polyester absorptive core, between two layers of a silver-coated high-density polyethylene mesh. In a moist environment, the silver nanocrystals (average crystal size of 15 nm) are released and improve microbial control on the wound. The silver coating in Acticoat consists of 0.2 to 0.3 mg silver per milligram of high density polyethylene, and is a binary alloy of silver (97%). It is less than 1 μm in thickness and engineered to change color in aqueous solution. The produced film coating is abrasion-resistant, nonadherent to the wound, and flexible. The extremely small size of the silver nanocrystals produces a very large surface area and the dressing core absorbs and

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accumulates moisture, thus maintaining a moist environment on the wound surface. The initial application is easy because it needs to be changed every 3 days (the antimicrobial barrier properties remain effective for a minimum of 3 days) and moistured once or twice a day with sterile water. In the instruction leaflet, there are only few contraindications and precautions listed.

Before human trials began, Acticoat was evaluated in vitro for the toxicity to mammalian tissue (minimal toxicity was found) for the release of silver in an aqueous environment (local concentration of silver ions 50 mg/L to 100 mg/L for up to 48 hours) and, in a porcine model, for adverse effects on wound healing (no adverse effects were detected). In these models, neutron activation analysis of plasma and urine revealed no evidence of systemic silver absorption with Acticoat.[19] In a clinical study, 30 burn patients were randomly treated with either 0.5% silver nitrate solution or Acticoat. The duration of the treatment was an average of 4 days. No adverse effects from use of Acticoat were found. The frequency of burn wound sepsis ($>10^5$ organisms/g tissue) was lower in the Acticoat group (5 vs. 16) and secondary bacteremia arising from infected burn wounds was also less frequent with Acticoat.[20] Plasma or urine samples for silver absorption were not obtained in this study. Yin et al.[21] tested the antimicrobial activity of Acticoat, 0.5% silver nitrate solution, 1% silver sulfadiazine cream, and 5% mafenid acetate solution on *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. Silver extracted from Acticoat had quite low minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against the tested bacteria, as had others, but Acticoat did kill the bacteria very quickly. In a controlled clinical study, Acticoat was tested as a donor site dressing and compared with Allevyn. Sixteen paired sites in 15 patients were evaluated. Acticoat-dressed donor sites took significantly longer for >90% re-epithelialization (14.5 ± 6.7 days vs. 9.1 ± 1.6), donor sites dressed with Acticoat had significantly worse scars at 1 and 2 months (the difference resolved by 3 months) and there was no difference in the incidence of positive bacterial cultures.[22]

We made a very similar observation in one 11-year-old boy whose donor sites were dressed with Acticoat. The wound healing was noticeably delayed (>2 weeks) compared with our current standard and it was difficult to remove Acticoat due to adherence to the wound ground on day 14 postsurgery. In contrast, Demling et al.[23] report an increased re-epithelialization of meshed skin grafts. In a porcine model of wound healing, a nanocrystalline silver-coated dressing promoted rapid metalloproteinase levels and enhanced cellular apoptosis.[3]

Particularly with regards to cultured skin substitutes, effective topical mixtures containing antimicrobial and antifungal agents are basic. For these reasons, alternatives have been investigated. Holder et al.[24] tested the antimicrobial activity of Acticoat, N-Terface, and Op-Site in strains of *S. aureus*, *P. aeruginosa*, *Candida albicans*, and divers gram-negative members of *Enterobactereicae*, isolated in the wounds of the burn patients. It was found that Acticoat has inherent antimicrobial properties, but, to be effective, hours of contact between Acticoat and the microorganisms are required. Acticoat also has a capacity to serve as an impenetrable barrier for all organisms tested in the study. Thomas et al.[25] also tested the antimicrobial activity of four silver-containing dressings (Acticoat, Actisorb Silver 220, Avance, and Contreet-H) against *S. aureus*, *E. coli*, and *C. albicans*. Acticoat produced the most rapid antimicrobial effect in vivo due to the rapid release of relatively large concentrations of highly active silver ions.

The antimicrobial efficacy of Acticoat wound dressing had been shown in several in vivo and in vitro studies. In contrast, there are few clinical studies published and no reports on adverse reactions or side effects with Acticoat therapy. We also failed to find any data on silver plasma and/or urine levels in humans treated with Acticoat.

Our patient was a young, healthy man who sustained 30% flame burns, 8% deep dermal. After 1 week of Acticoat treatment, his wounds were found to be healing rapidly and without pathologic findings. The patient never complained about discomfort or pain in the burn area. The first striking symptom was his facial appearance: a grayish discoloration and pale-bluish lips, gradually increasing. The evident argyria-like face discoloration in our patient was the final clue to perform a plasma and urine silver check. The phenomenon described does not match argyria, which is a permanent disorder caused by silver deposition in the skin's microvessels in patients who are exposed to chronic silver toxicity.[26,27]

CONCLUSION

In our case, it has to be assumed that the transient skin discoloration was related to the acutely elevated blood silver levels. The liver function abnormalities observed in our patient can only be an effect of acute silver toxicity because there was no other cause for liver damage; the patient was not taking drugs or alcohol and was not given potentially hepatotoxic medication. He was not dehydrated and had normal renal function at all times.

This evidentiary assumption that silver released from Acticoat was the cause for our patient's problems is supported by the fact that the clinical argyria-like symptoms disappeared and liver function tests returned to normal after the Acticoat local treatment was discontinued.

This is the first report on silver toxicity in a patient with 30% burns who received Acticoat for local treatment. Due to substantial experiences with adverse reactions and side effects to SSD, it is appropriate to keep the possibility of the toxic silver effect in burn patients treated with Acticoat silver-coated wound dressing in mind. The silver levels in plasma and/or urine should be monitored.

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An in vitro analysis of the antimicrobial properties of 10 silver-containing dressings

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The variation in the structure and properties of the many silver dressings now on the market might be expected to affect their antimicrobial properties. This study, which follows up a paper published last March, compares their performance

Silver-containing dressings, indicated primarily for the treatment or prevention of soft-tissue infections, depend on the ability of low concentrations of silver ions to kill a broad spectrum of microorganisms. Some of the new materials have been developed to carry and release silver in a controlled fashion. In others cases, a silver compound has been added to an existing product.

Given the considerable variation in the structure, composition and silver content of these new preparations, marked differences might also be anticipated in their ability to release silver in sufficiently high concentrations to exert a significant antimicrobial effect. This could have important implications for the dressings' clinical performance.

We have already described how the antimicrobial activity of four silver-containing dressings was compared in a laboratory-based study using three tests.[1] The dressings were: Acticoat (Smith and Nephew), Actisorb Silver 220 (Johnson and Johnson), Avance (SSL International) and Contreet-H (Coloplast).

This follow-up paper reports the results of further tests conducted in an identical manner on an additional six silver-containing dressings, together with a non-woven swab (negative control) and Acticoat (positive control).

Materials and methods

The six new dressings are described below, using the limited information provided by the manufacturers.

Arglaes (Medline)

Contains a mixture of an alginate powder and an inorganic polymer containing ionic silver. The alginate absorbs moisture to form a gel, and the silver complex breaks down in a controlled fashion to liberate ionic silver into the wound.

Aquacel Ag (ConvaTec)

Comprises a fleece of sodium carboxymethylcellulose (CMC) fibres containing 1.2% ionic silver. The dressing absorbs moisture to form a gel, binding sodium ions and releasing silver ions.

Calgitrol (Magnus Bio-Medical Technologies)

A silver alginate dressing comprising an absorbent foam sheet, one surface of which is coated with an alginate matrix containing ionic silver, together with a 'cleanser, moisturiser and a superabsorbent starch co-polymer'.

Contreet Ag (Coloplast)

A polyurethane foam dressing containing silver in a so-called 'hydroactivated' form, which is released as the foam absorbs liquid.

Silverlon (Argentum Medical)

A knitted fabric dressing, silver-plated by means of a proprietary autocatalytic electroless chemical (reduction-oxidation) plating technique. This coats the entire surface of each individual fibre, resulting in a very large surface area for the release of ionic silver.

Silvasorb (Medline)

Composed of a synthetic, polyacrylate, hydrophilic matrix in which microscopic silver-containing particles are

dispersed or suspended. On exposure to moisture, silver is released in a controlled fashion.

Test organisms

This study set out to compare the antimicrobial properties of the dressings rather than investigate the antimicrobial activity of silver itself.[1] Three standard organisms were therefore used:

- Gram-positive: *Staphyloccocus aureus* (ATCC 6538P)
- Gram-negative: *Escherichia coli* (ATCC 8739)
- A yeast: *Candida albicans* (ATCC 2091).

Test methods

Three different methods were designed to compare various aspects of performance. Full details are given in the original article.[1]

- **Zone of inhibition** Samples of each dressing were placed on agar plates inoculated with 0.2ml of a log-phase broth culture of each test organism. After incubation, the plates were examined for the presence of a zone of inhibition. If one was detected, the width was measured and the dressing was removed from the agar, placed on another agar plate and seeded as before with the same microorganism. This process was repeated a maximum of seven times or until no further zone of inhibition was produced during the previous test.

- **Challenge testing** 0.2ml of a log-phase culture of each microorganism was added to portions of each dressing measuring 40x40mm. The inoculated dressings were incubated for two hours, then transferred into 10ml of 0.1% peptone water (Oxoid) and vortexed to remove any remaining viable organisms. Serial dilutions were performed in triplicate on each extract, and the number of viable organisms present determined using a standard surface counting technique.

If viable organisms were recovered, the test was repeated as before using a four-hour contact period, and then again with a 24-hour contact period.

If no organisms were detected on a particular dressing after two hours, the dressing was placed in 10ml of tryptone soya broth (TSB) to detect very low levels of residual contamination.

As no inactivator for silver was used during this test, it is possible that any remaining low concentrations of silver ions present could have prevented the recovery of these organisms, potentially resulting in a false negative result.

- **Microbial transmission test** Here, a strip of dressing forms a bridge between two separate agar blocks in a Petri dish, one of which is sterile and the other inoculated with the test organism. This test determines the bacteria's ability to survive on the dressing surface and migrate along it from the contaminated agar to the sterile agar. A positive result suggests that it is possible that microorganisms could be transported laterally out of a contaminated wound onto the surrounding skin, or potentially move in the opposite direction from the intact skin into the wound itself.

Silver content

Samples of each dressing were sent to Sheffield Analytical Services to determine the total extractable silver content of each dressing by inductively coupled plasma optical emission spectroscopy (ICP-OES).

Results

Zone of inhibition test

The results of this method for the three test organisms are summarised in Table 1, which also includes results from the original paper.[1] There was considerable variation in ability to inhibit growth of the three test organisms. To further facilitate comparisons, a simple scoring system is also included. A dressing gets three points for each appearance in group A, two points for each appearance in group B and no points for each appearance in group C. The sum of these scores produces a very crude measure of the dressings' overall performance in this test.

Microbial challenge test

The results of this test are summarised according to the dressings' ability to produce a marked antimicrobial effect, arbitrarily defined as a 10^3 reduction in the number of viable organisms present at each time interval (Table 2). A similar scoring system to that outlined above has been devised to facilitate later comparisons. Due to the physical nature of the Arglaes material, it was not possible to include it in this series of tests.

Microbial transmission test

Results are summarised in Table 3. Again, both Tables 2 and 3 include results from the first paper.[1] Other than the control material, the only test samples to show any evidence of microbial transfer were Actisorb Silver 220 and Avance.

As previously discussed,[1] in the case of Actisorb Silver 220, microbiological migration occurred on only one sample, and probably took place across the nonwoven fabric outer sleeve of the dressing. No transfer occurred when only the inner core of the dressing was examined.

Evidence of transmission of bacteria was clearly visible on all three samples of Avance, as shown by prolific bacterial growth around the ends of the dressing on the surface of the sterile agar.

No transfer of *Candida albicans* took place on any of the sample tested including the control, which made the tests invalid.

Silver content of dressings

The total silver content of each dressing included in both papers is shown in Table 4 ranked by silver content, which indicates that major differences exist between the these products, with values ranging from 546 to 1.6mg/ $100cm^2$. Also included are the total scores achieved by each dressing in the various laboratory tests.

Discussion

The test methods were designed to compare the performance of the dressings under different simulated conditions of use.

The zone of inhibition method simulates the use of the products on moist or lightly exuding wounds and predicts the dressings' ability to kill or prevent bacterial growth in this situation.

In order to exert a significant antimicrobial effect in this test, a dressing must first absorb moisture from the agar to activate or release the silver held within its structure. This silver, in the form of silver ions, must then diffuse back down into the agar to exert its antimicrobial action.

The microbiological challenge test provides an indication of each dressing's ability to kill or prevent growth of predetermined numbers of bacteria applied directly in the form of a suspension, and thus to some extent reflects what may occur within dressings applied to more heavily exuding wounds.

The third test determines the bacteria's ability to survive and be transmitted along the dressing surface.

It was anticipated from the outset that the ability to exert a significant antimicrobial effect would be directly related to the total amount of silver present. The very crude scoring system described above seems to support this view. There is clearly a very strong association between the dressings' measured silver content and the scores they achieved in the laboratory tests, although two results for Silverlon and Contreet Ag require further comment.

Although it scored highly overall, the somewhat poor performance of Silverlon against *Staphylococcus aureus* in the challenge test was surprising given that it contained by far the highest concentration of silver of any of the dressings examined (four to five times the amount of the next two highest products). Similarly, although Contreet Ag performed well in some tests, it was disappointing in others.

While total silver content is important, other factors also influence a dressing's ability to kill microorganisms. These include the distribution of the silver within the dressing (whether it is present as a surface coating or is dispersed through the structure), its chemical and physical form (whether it is present in a metallic, bound or ionic state) and the dressing's affinity for moisture — a prerequisite for the release of active agents in an aqueous environment. Products in which the silver content is concentrated on the dressing surface rather than 'locked up' within its structure performed well, as did those in which silver was present in the ionic form.

Calgitrol, which contains a high concentration of silver, performed very well in all tests. This is probably because the silver, already in the ionic form, is concentrated on the dressing surface in a hydrophilic coating, which facilitates its rapid release.

Contreet Ag and Contreet-H, although containing broadly similar concentrations of silver, performed very differently in the first two tests. In the zone of inhibition test the hydrocolloid performed well, unlike Contreet Ag. In contrast, in the challenge test the foam-based Contreet Ag markedly outperformed the hydrocolloid.

The reasons for this are not entirely clear but may be related partly to differences in the fluid-handling characteristics of the two dressings. Under the test conditions, absorbency of Contreet Ag may be such that it created a suction gradient, continuously drawing fluid out of the agar and inhibiting the movement of solution containing silver ions in the reverse direction. Also, the foam had a tendency to curl away from the agar plate. In the challenge test, however, the organisms applied directly to the foam were destroyed by the silver ions released within its structure. This test probably more closely reflects the dressing's performance in the management of more heavily exuding wounds.

Aquacel contains ionic silver in a hydrophilic fibrous fleece. This material's fluid affinity is such that it was readily capable of drawing moisture out of the agar, which then released the silver ions. This enabled the dressing to exert significant antimicrobial activity on extended incubation, despite the relatively modest silver content.

Silvasorb, which also contains a relatively low concentration of ionic silver, showed broadly similar activity to Aquacel due to its hydrophilic structure.

Actisorb Silver 220, which contains low concentrations of metallic silver, showed evidence of only very limited antimicrobial activity.

Avance, which has the lowest silver content of the products examined, showed no evidence of any antimicrobial effect in any of the tests.

Considerable caution must be exercised when extrapolating the results of this or any laboratory-based study to the clinical situation as many factors determine a dressing's acceptability or clinical effectiveness, which may not become apparent in a laboratory model. For example, this study made no attempt to compare the fluid-handling properties of the various products or to determine their tissue compatibility or potential cytotoxic effects. All these issues were discussed at length.[1] Nevertheless, we believe our results may provide useful information to clinicians on one key aspect of the performance of this relatively new class of products.

This study was partly supported by Smith and Nephew

More detailed data on the results of the zone of inhibition and challenge tests are available from the author

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1 Thomas, S., McCubbin, P. A comparison of the antimicrobial effects of four silver-containing dressings on three organisms. J Wound Care 2003; 12: 3, 101-107.

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Table 1. Summary of zone-of-Inhibition data

	S. aureus	*E. coli*	*C. albicans*
Group A (score 3)			
Products that show evidence of sustained activity over two or more days	Acticoat Aquacel Ag Calgitrol Ag Contreet-H Silverlon	Acticoat Calgitrol Ag Contreet-H Hydrocolloid Silverlon	
Group B (score 2)			
Products that produce a well-defined zone of inhibition at one time interval	Arglaes Power Silvasorb	Aquacel Ag Arglaes Powder	Acticoat Arglaes Powder Calgitrol Ag Contreet-H Silvasorb Silverlon
Group C (score 0)			
Products that produce no well-defined zone of inhibition in this test	Actisorb Silver 220 Avance Contreet Ag	Actisorb Silver 220 Avance Contreet Ag Silvasorb	Actisorb Silver 220 Aquacel Ag Avance Contreet Ag

Table 2. Summary of microbial challenge test results

	S. aureus	*E. coli*	*C. albicans*
Group A (score 4)			
Products that demonstrate marked antibacterial activity after two hours' incubation	Acticoat Calgitrol Ag	Acticoat Calgitrol Ag Contreet Ag Silverlon	Acticoat Calgitrol Ag Contreet Ag Silverlon
Group B (score 3)			
Products that demonstrate marked antimicrobial activity after four hours' incubation	Silverlon	Contreet-H Aquacel Ag Silvasorb	
Group C (score 2)			
Products that demonstrate marked antimicrobial activity after 24 hours' incubation		Actisorb Silver 220	
Group D (score 1)			
Products that demonstrate limited evidence of antimicrobial activity after 24 hours' incubation	Aquacel Ag Contreet-H Contreet Ag Silvasorb		Contreet-H Aquacel Ag Silvasorb
Group E (score 0)			
Products that demonstrate no convincing evidence of antimicrobial activity even on prolonged incubation	Actisorb Silver 220 Avance	Avance	Actisorb Silver 220 Avance

Table 3. Microbial transmission test

	S. aureus	*E. coli*	*C. albicans*
	Transfer ±	Transfer ±	Transfer ±
Acticoat	-	-	-
Actisorb Silver 220	+	+	-
Actisorb Silver 220*	-	-	-
Avance	+++	+++	-
Contreet-H	-	-	-
Control	+++	+++	-
Acticoat	-	-	-
Aquacel Ag	-	-	-
Calgitrol Ag	-	-	-
Contreet Ag	-	-	-
Silvasorb	-	-	-
Silverlon	-	-	-
Control	+++	+++	-

Each + indicates the results for a single test strip
*Only the inner core of Actisorb Silver 220 was used

Table 4. Silver content of the dressings

Product	Batch no.	Ag content ($mg/100cm^2$)	Total performance scores
Silverlon	102502-01	546	19
Calgitrol Ag	131-71	141	20
Acticoat	010814A-08 020214A	109 101	20
Contreet Ag	74853.01	47	9
Contreet-H	315768 267462 344046	31.2 32.4 31.4	13
Aquacel Ag	2H55863	8.3	10
Silvasorb	02082001	5.3	9
Actisorb Silver 220	0138-03 0135-04	2.9 2.4	2
Avance	01106947	1.6	0
Arglaes powder	527027	6.87mg/gram	

PROPERTIES OF ALGINATES

BY

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ALGINATE INDUSTRIES LIMITED

WALTER HOUSE · BEDFORD STREET · STRAND · LONDON · W.C.2

Exhibit K

that guluronic acid residues as well as those of mannuronic acid were present. This has since been confirmed by other workers[11, 12]. It is not quite certain whether polymannuronic acid and polyguluronic acid chains exist independently in alginic acid, or whether the two uronic acids occur in the same chains. It has been found that alginates can be fractionated by precipitation with manganese[13] salts and with potassium chloride[14], and that the two types of polyuronide are concentrated in the different fractions[15], but on the other hand, di- and triuronides apparently containing both mannuronic and guluronic acid have been isolated from the hydrolysis products of alginic acid[16].

Manufacturers and users of alginates have long been aware of quantitative differences in the behaviour of alginates from different species of algae: it is now known that the proportions of mannuronic and guluronic acid vary from one species to another and this may go a long way towards accounting for these differences. However, with the present state of our knowledge the different alginates must still be referred to by their origin rather than by proportions of uronic acids.

It must be emphasised that the physical and chemical properties of alginates from different sources are the same from a qualitative point of view, and the differences are to be found only when precipitation reactions and the physical properties of precipitated or mixed salt alginates are examined quantitatively. This is to be expected from the similarity of structures of the two polyuronides which they contain in differing proportions.

A study of sodium alginates from different sources was made by Vincent, Goring and Young[17]. Properties investigated included ionic mobilities and sedimentation constants, and the samples covered a wide range of molecular weight as shown by the viscosities of their solutions. No significant variation in the properties examined could be related to the origin of the products. It is therefore reasonable to continue to use the name alginic acid for the products from different species, but published work giving quantitative results without reference to seaweed origin should be treated with caution. The degree of polymerisation, which can be varied by adjusting the methods of extracting the alginates from the different algae, can also modify other properties quantitatively.

that guluronic acid residues as well as those of mannuronic acid were present. This has since been confirmed by other workers[11, 12]. It is not quite certain whether polymannuronic acid and polyguluronic acid chains exist independently in alginic acid, or whether the two uronic acids occur in the same chains. It has been found that alginates can be fractionated by precipitation with manganese[13] salts and with potassium chloride[14], and that the two types of polyuronide are concentrated in the different fractions[15], but on the other hand, di- and triuronides apparently containing both mannuronic and guluronic acid have been isolated from the hydrolysis products of alginic acid[16].

Manufacturers and users of alginates have long been aware of quantitative differences in the behaviour of alginates from different species of algae: it is now known that the proportions of mannuronic and guluronic acid vary from one species to another and this may go a long way towards accounting for these differences. However, with the present state of our knowledge the different alginates must still be referred to by their origin rather than by proportions of uronic acids.

It must be emphasised that the physical and chemical properties of alginates from different sources are the same from a qualitative point of view, and the differences are to be found only when precipitation reactions and the physical properties of precipitated or mixed salt alginates are examined quantitatively. This is to be expected from the similarity of structures of the two polyuronides which they contain in differing proportions.

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Alginate Solutions

General Discussion

The soluble alginates have properties typical of hydrophilic colloids. For example; their solutions are much more viscous than those of simple substances at the same concentration; alginates can be separated from substances of low molecular weight by dialysis; and the process of drying out a solution is readily reversible. (As the alginate is molecularly dispersed, it is reasonable to refer to a solution of the alginate in water). It is now usual to refer to alginates and similar substances as polyelectrolytes. They have large molecules and in solution carry an electric charge by reason of their electrolytic dissociation.

It is generally found that an alginate is either practically insoluble or is completely miscible with water, unlike substances of low molecular weight where the solid will exist in equilibrium with a saturated solution containing a considerable amount of the dissolved solid. This type of behaviour is usual with high polymers, but it should be noted that in some cases there can be separations of fractions of different degrees of polymerisation, the more highly polymerised material remaining undissolved in a solution of the lower polymers. It has also been found that there can be some additional separation into fractions having higher or lower ratios of mannuronic to guluronic residues.

In many cases an alginate can quite definitely be described as insoluble, as no trace of dissolved material can be detected when the alginate is mixed with water. This is the case with most metallic alginates. Others, such as sodium alginate, are definitely very soluble in water, but in dealing with mixed salts, or with limiting concentration of precipitants it is not possible to give the exact limiting conditions for solubility. Difficulties in the way of quantitative examination are:

(*a*) The high viscosity of even moderately concentrated alginate solutions, which makes the separation of two phases difficult.

(*b*) The highly swollen state of precipitated alginates.

(*c*) The extremely slow attainment of equilibrium between phases.

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Figures for limits are therefore only approximate. It is sometimes difficult to be certain whether an alginate is completely dissolved or not. For example, mixtures of sodium alginate and water in all proportions ranging from water through viscous solutions and pastes to hard solids can be made. Whether these are all one phase systems, or whether in some two phases are present, is uncertain.

Alginates are essentially hydrophilic and the simple alginates are insoluble in common non-aqueous solvents, although sodium alginate can be dissolved, for example, in molten urea. The addition of water miscible liquids, such as alcohol, to aqueous solutions of most alginates will cause precipitation. The amount of liquid which can be added without causing precipitation depends on the base combined with alginic acid. While sodium alginate is precipitated by the addition of 20% to 30% of alcohol, amine salts, particularly those of the higher amines are soluble in mixtures of alcohol and water containing a high proportion of alcohol. There is now available an amine alginate (Collatex P) which is soluble in lower alcohols but insoluble in water. Ammonium alginate requires more alcohol for precipitation than sodium alginate, but less than that of most of the salts of organic bases. The exact amount required depends in all cases on the concentration of the solution and the degree of polymerisation of the alginate.

In the following discussion of solubility relations, it is the solubility in water that is meant in each case.

Solubilities of Individual Alginates

Alginic acid is insoluble. Considering its chemical nature this is at first sight surprising, and here a comparison with cellulose and pectin is illuminating (see Fig. 1, page 4). The X-ray examination of both alginic acid and cellulose has shown them to be largely in a crystalline state, so that they have very regular structures, affording ample opportunity for hydrogen bonding between polymer chains at regular intervals. On the other hand pectin is the partial methyl ester of pectic acid, the carboxyl groups being esterified at random. It is therefore understandable that the energy required to separate the molecular chains, the first step towards solution, is much

greater for alginic acid and cellulose than for pectin. Pectin is in fact completely miscible with water, but removal of the methyl group by hydrolysis gives pectic acid, insoluble in water, and similar in properties to alginic acid. In the same way some derivatives of cellulose and of alginic acid, in which irregularities in the chain reduce the opportunity for crystallisation, are soluble in water. Examples are the partial methyl ether of cellulose and the partial propylene glycol ester of alginic acid.

The presence of the acid groups in alginic acid affords a very easy method of bringing it into solution, as by the formation of a highly ionised salt, electrostatic forces can shift the free energy balance in favour of solution. In titrating alginic acid with sodium hydroxide, about three-quarters of the carboxyl groups have to be neutralised to bring about solution. This takes place at about pH 3.5. The transition is quite sharp. For example Saric and Schofield[25] found that at pH 3.6 all their alginate was dissolved, but at pH 3.3 the whole could be centrifuged out as alginic acid.

The salts of the alkali metals, ammonia, and many organic bases are soluble in water while those of most of the di- and polyvalent metals are insoluble. The insolubility of these alginates is probably due to a combination of forces, but it is understandable that, even if completely ionised, divalent cations could keep the alginate ions sufficiently closely associated to prevent solution. The valency of the base is not the sole deciding factor as magnesium alginate is soluble, while silver alginate is insoluble.

The solubilities of some of the common alginates and their colours are given in Table 1. In the last column some procedures for obtaining clear solutions, free from precipitated salts, are included. These methods involve chemical transformation of the alginate, but it is sometimes useful to prepare solutions in this way rather than starting with a soluble alginate. Solution brought about by adding ammonium hydroxide is readily reversed by removal of the ammonia so that insoluble films can be formed by evaporation. Insoluble alginates can also be brought into solution by reaction with the alkali salt of an anion which forms an insoluble

salt with the metal combined with the alginate, but in this case the resulting solution contains the insoluble salt as a suspension or sediment.

TABLE I

Alginate Salt	Colour	Solubility in Water	Dissolved by Adding
Sodium, potassium ammonium, magnesium	White	Soluble	
Triethanolamine	Pale Yellow	Soluble	
Calcium	White	Insoluble	Sodium metaphosphate
Aluminium	White	Insoluble	Ammonium hydroxide
Zinc	White	Insoluble	Ammonium hydroxide and ammonium salt
Copper	Blue	Insoluble	Ammonium hydroxide
Chromium	Grey-green	Insoluble	
*Iron (ferrous)	Pale-green	Insoluble	
Iron (ferric)	Brown	Insoluble	
Silver	White (darkens in light)	Insoluble	Ammonium hydroxide

* There has been some doubt about the solubility of ferrous alginate—for further discussion see Page 29.

Dissolving Soluble Alginates

Soluble alginates are easily and quickly dissolved in water with the aid of a high speed stirrer. Other methods can be used but are not so rapid. Details are given in the A.I.L. leaflets " Dissolving Alginates " and " Dissolving Readily Soluble Alginates."

It is advisable to use soft water when preparing alginate solutions. This is essential when dilute solutions are being made; the higher the ratio of water to alginate, the more serious would be small amounts of dissolved calcium in the water. The extreme case of dilution arises when an alginate is being washed out of, for example, a fabric, and it is useful then to include sodium metaphosphate or carbonate in at least one lot of the wash water.

TABLE II

Compatibilities of Alginates in Solution

A. COMPATIBLE

Some common substances, high proportions of which can be included in aqueous alginate solutions without causing precipitation.

Polyhydric alcohols	Ethylene glycol, glycerol, sorbitol, mannitol.
Carbohydrates	Simple sugars, starch, soluble cellulose derivatives.
Gums	Acacia, tragacanth, locust bean, guar, karaya, carrageenin, pectin.
Salts	Most salts of the alkalis, ammonium and magnesium, e.g. sodium sulphate, sodium carbonate, borax, magnesium sulphate.
Dyestuffs	Directs, vats, solubilised vats, Rapidogens, many acid dyes, pigment dyestuffs and binders.
Proteins	Gelatine, egg albumen, casein, vegetable proteins (calcium assumed absent or sequestered).
Miscellaneous	Urea, phenol, emulsified oils and resins, boric acid.

B. INCOMPATIBLE

Some substances which even in low concentration precipitate alginates from solution.

Salts	Soluble ionised salts of alkaline earths and heavy metals.
Acids	Strong acids (sufficient to bring the pH to below 3.5 in alginate salt solutions, 2.5 in propylene glycol solutions.
Organic substances	Cationic detergents, positively charged colloids.

Precipitation of Alginates without Chemical Change

Although precipitation is generally due to the formation of a water insoluble alginate, some substances can bring about precipitation without chemical change; the action of water miscible non solvents for alginates has already been mentioned (page 10). Precipitation can also be brought about by moderate or high concentrations of some simple electrolytes. This is presumably a salting out effect similar to that which takes place with many other colloids, but quantitatively the effect of different salts is difficult to understand. For example, certain types of sodium alginate are precipitated by N (5.8 gm./100 ml.) sodium chloride, but will dissolve in 3 N (21.3 gm./100 ml.) sodium sulphate.

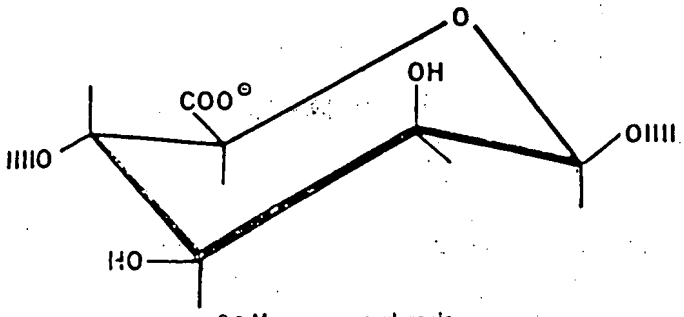
Kelco
DIVISION OF MERCK & CO. INC.

Kelco Algin/hydrophilic derivatives of alginic acid for scientific water control

Second Edition

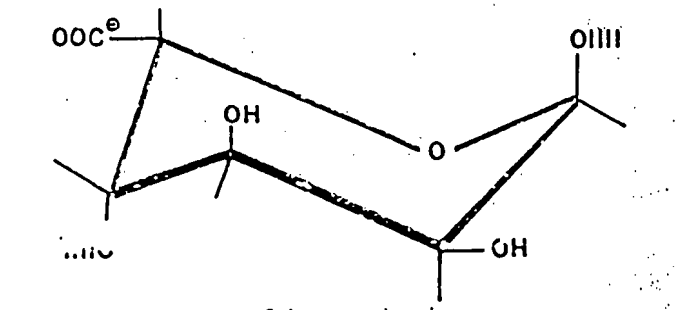
Exhibit L

Figure 4-A. Conformation of mannuronic acid.



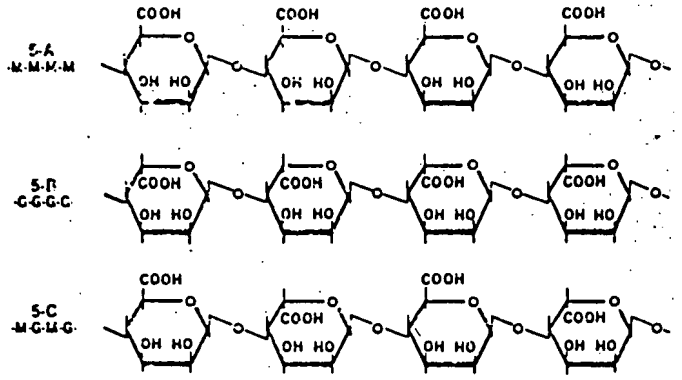
β-D-Mannopyranosyluronic acid unit—C1 conformation

Figure 4-B. Conformation of guluronic acid.



α-L-Gulopyranosyluronic acid unit—1C conformation

Figure 5-A-C. Structure of the polymer segments contained in alginic acid.



STRUCTURE

Algin, the polysaccharide extracted from the brown seaweeds, *Phaeophyceae*, has only recently been chemically identified.

Although purified alginic acid was prepared by Krefting in 1896, the first report on its structure was made in 1930 by Nelson and Cretcher, who claimed it was a D-mannuronic acid polymer. Later investigation by Hirst and co-workers determined alginic acid to be composed of D-mannuronic acid connected by β1→4 linkages (*Hirst et al. 1939*). In 1955, Fischer and Dörfel, using paper chromatography, determined that L-guluronic acid was also present as a major component of alginic acid. Vincent (*1960*) and Hirst, Percival, and Wold (*1964*) showed that at least some of the alginic acid molecules contained both mannuronic acid (Figure 4-A) and guluronic acid (Figure 4-B) by use of partial acid hydrolysis to isolate oligomers containing both uronic acids.

Improvements in the techniques for the hydrolysis, separation, and analysis of alginic acid have allowed accurate determinations of the composition of alginic acid from different sources to be made. Table 2 shows the composition of alginic acid obtained from commercially important brown algae.

The presence of three kinds of polymer segments in alginic acid from various brown algae has been shown by mild acid hydrolysis (*Haug et al. 1966, 1967a, and 1967b*). One segment consists essentially of D-mannuronic acid units (Figure 5-A); a second segment consists essentially of L-guluronic acid units (Figure 5-B); and the third segment consists of alternating D-mannuronic acid and L-guluronic acid residues. (Figure 5-C).

The proportions of the three polymer segments in alginic acid samples from different sources have been determined by Haug and co-workers (*1966 and 1967a*) using partial acid hydrolysis to separate the alginic acid into homopolymeric and alternating segments. Penman and Sanderson (*1972*) have determined the proportion of polymannuronic acid and polyguluronic acid segments by p.m.r. spectroscopy. Table 3

Table 2
Mannuronic Acid (M) and Guluronic Acid (G) Composition of Alginic Acid Obtained from Commercial Brown Algae

Species	Mannuronic Acid Content (%)	Guluronic Acid Content (%)	M/G Ratio	M/G Ratio Range
Macrocystis pyrifera	61	39	1.56[a]	–
Ascophyllum nodosum	65	35	1.85 (1.1)[a]	1.40-1.95[b]
Laminaria digitata	59	41	1.45[a]	1.40-1.60[b]
Laminaria hyperborea (stipes)	31	69	0.45[a]	0.40-1.00[b]
Ecklonia cava and Eisenia bicyclis	62	38	1.60[a]	–

(a) Data of Haug (*1964*) and Haug and Larsen (*1962*) for commercial algin samples. Of the two ratios shown for *Ascophyllum nodosum*, the algin sample manufactured in Canada has the higher M/G value; the lower ratio corresponds to a European sample.

(b) Data of Haug (*1964*) showing the range in composition for mature algae collected at different times at each of several locations.

Table 3
Proportions of Polymannuronic Acid, Polyguluronic Acid, and Alternating Segments in Alginic Acid Isolated from Brown Algae[a]

Source	Polymannuronic Acid Segment (%)	Polyguluronic Acid Segment (%)	Alternating Segment (%)
Macrocystis pyrifera	40.6	17.7	41.7
Ascophyllum nodosum	38.4	20.7	41.0
Laminaria hyperborea	12.7	60.5	26.8

(a) Data of Penman and Sanderson (1972)

shows the proportions of polymannuronic acid, polyguluronic acid, and alternating segments in three commercial alginic acid samples.

The differences in composition and fine structure indicated in Tables 2 and 3 account for the differences in properties and functionality of alginates isolated from different species of brown algae.

The theoretical equivalent weight of alginic acid is 176, but the bound water within the molecule results in measured values close to 194 (Haug 1964).

The dissociation constant for alginic acid depends on the ratio of mannuronic acid to guluronic acid. Haug (1964) has reported the following dissociation constants:

Acid	pKa
Mannuronic Acid	3.38
Guluronic Acid	3.65

Recently it has been shown, using p.m.r. spectroscopy and model building with a computer, that alginic acid salts, such as sodium alginate, in aqueous solution are highly hydrated polyelectrolytes in the extended ribbon conformation.

X-ray diffraction studies on fibers of alginic acid and polarized infrared spectroscopy of oriented films have resulted in information on the crystalline structure of polymannuronic acid and polyguluronic acid.

The shape of the polymannuronic acid chain is similar to that found in other $\beta 1 \rightarrow 4$ linked hexosans such as cellulose. The mannuronic acid is in the C1 conformation and, therefore, di-equatorially linked (Figure 6-A). Polymannuronic acid is a flat, ribbon-like molecule (Figure 7-A), the conformation of which appears to be stabilized by the formation of an intra-molecular hydrogen bond between the hydroxyl group on carbon 3 of one unit and the ring oxygen atom (O_5) of the next sugar unit in the chain (Atkins et al. 1971). The chains themselves are bonded into sheets by means of hydrogen bonds formed between the hydroxyl of the carboxyl group and the oxygen atom on carbon 3 in sugar units in parallel chains and between the axial hydroxyl group on carbon 2 and the oxygen atom of the carboxyl group in antiparallel chains.

Figure 6-A. Repeating unit of polymannuronic acid.

The glycosidic linkage is 1e→4e, i.e., di-equitorial.

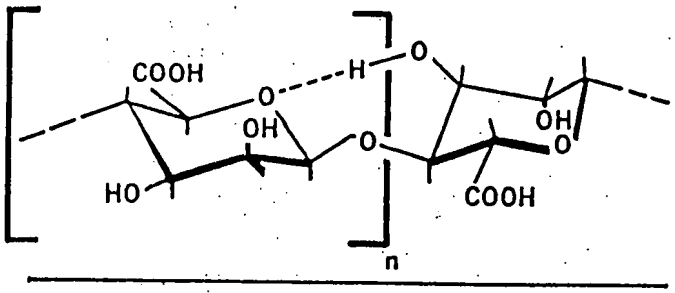
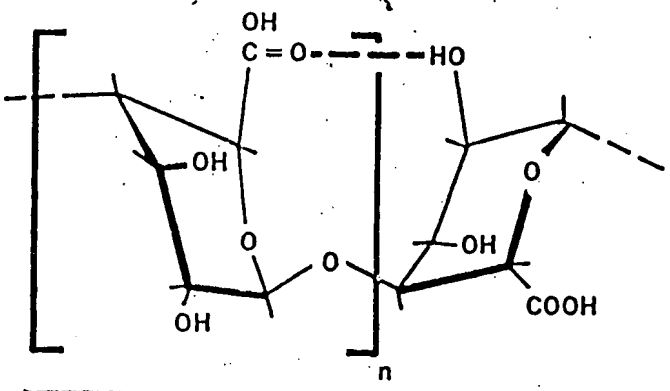


Figure 6-B. Repeating unit of polyguluronic acid showing the difference in conformation.

The glycosidic linkage is 1a→4a, i.e., di-axial.



The shape of the polyguluronic acid chain is quite different from that of polymannuronic acid. Polyguluronic acid is a buckled, ribbon-like molecule (Figure 7-B) in which the guluronic acid is in the 1C conformation and, therefore, di-axially linked (Figure 6-B). The buckled, ribbon-like conformation is stabilized by an intramolecular hydrogen bond between the hydroxyl group on carbon 2 and the oxygen atom of the carboxyl group in adjacent units. The interchain bonds are more complicated than in the case of polymannuronic acid and involve water molecules. A water molecule is in such a position that it functions twice as a hydrogen bond donor and twice as an acceptor, the hydrogen bonds so formed being in the range of 2.7Å-2.9Å. In accord with den-

IV Toxicity, Regulatory, and Environmental Information

The toxicological properties of alginates have been extensively investigated and summarized *(McNeely and Kovacs 1975; Anon. 1972).*

One of the earliest studies investigated the digestibility of sodium alginate in male albino rats during a 10-day period. The results indicated that the digestibility of sodium alginate ranged from 3 to 88% depending upon the level fed *(Nilson and Lemon 1942; Nilson and Wagner 1951).* Sodium alginate and propylene glycol alginate were incorporated in the diets of rats, mice, chicks, cats, and guinea pigs for prolonged periods without any deleterious effects.

In a series of separate experiments, Morgan *(1959)* placed 5% sodium alginate, standardized sodium alginate (DARILOID), and propylene glycol alginate in the diet of 40 male and 40 female albino rats for two years. Neither the parent generation (the F_1 generation) nor their progeny (the F_2 generation) showed any adverse effects from this prolonged exposure to alginates in their diet *(Johnston et al. 1964).*

An investigation by Woodard *(1959)* indicated that incorporating 5 to 15% sodium alginate or propylene glycol alginate in the diet of purebred beagle dogs for the period of one year caused no harmful effects.

Since no satisfactory data were reported in the literature on the acute oral toxicity (LD-50) of alginates, Kelco recently commissioned the Woodard Research Corporation to conduct studies on two algin compounds. The results indicated that the administration of 5 g/kg body weight to rats within a 24-hour period caused no mortalities nor signs of toxicity *(Knott 1972).*

A recent study indicated that the subcutaneous injection of mice with alginic acid caused no carcinogenic activity *(Epstein et al. 1970).* An investigation of the effect of degraded and undegraded sodium alginate on the colon of guinea pigs indicated that alginate did not cause ulcerative colitis either in its degraded or undegraded form *(Watt and Marcus 1971).* The investigation of the metabolic pathway of propylene glycol alginate indicated that the alginate moiety of the molecule remains unabsorbed, while the non-toxic propylene glycol produced by the hydrolysis of the propylene glycol alginate is absorbed through known metabolic pathways *(Sharratt and Dearn 1972).* Sodium alginate and propylene glycol alginate have also been shown by recent investigations to possess no eye or skin irritation properties *(Johnston 1972a and 1972b).*

In summary, numerous studies have attested to the high level of safety of alginates in food use.

Government Regulations and Labeling Requirements

Ammonium alginate, calcium alginate, potassium alginate, and sodium alginate are included in a list of stabilizers that are generally recognized as safe (GRAS) under 21 CFR 121.101. Propylene glycol alginate is approved as a food additive under 21 CFR 121.1015 for use as an emulsifier, stabilizer, or thickener in foods in accordance with good manufacturing practice.

The use of the edible salts of alginic acid, as well as propylene glycol alginate, is approved in all appropriate standard of identification regulations. In these standards, alginates are either specifically mentioned by their common and usual name or are included under the provision of "safe and suitable optional ingredients." For maximum allowable usage levels, each regulation must be consulted separately.

Propylene glycol alginate is approved for use in defoaming agents (21 CFR 121.1099), in coating on fresh citrus fruit (21 CFR 121.1179), as an inert pesticide adjuvant (40 CFR 180.1001), and as a component of paper and paperboard in contact with aqueous and fatty foods (21 CFR 121.2526).

Ammonium alginate and sodium alginate are approved for use as boiler water additives under 21 CFR 121.1088. The edible salts of alginic acid are also approved for use as a component of paper and paperboard in contact with aqueous and fatty foods under 21 CFR 121.2526, specifying that substances generally recognized as safe (GRAS) can be used.

KELGIN W and KELCOSOL, both of which are sodium alginates, are approved by the United States Public Health Service as coagulant aids for water treatment at maximum concentrations of 2 mg/l *(Anon. 1970).*

The Food Chemicals Codex contains monographs on alginic acid, ammonium alginate, calcium alginate, potassium alginate, sodium alginate, and propylene glycol alginate *(National Research Council 1972).* Monographs on sodium alginate and alginic acid are also included in the National Formulary *(Anon. 1975).*

Alginic acid and its edible salts, as well as propylene glycol alginate, are included in the approved emulsifier/stabilizer lists published by the EEC and the Council of Europe. The FAO/WHO Joint Expert Committee established an Average Daily Intake (ADI) limit of 50 mg/kg for alginic acid and its edible salts and 25 mg/kg for propylene glycol alginate.

Biological Oxygen Demand

Biological and chemical oxygen demand data for KELGIN MV (sodium alginate), SUPERLOID (ammonium alginate), and KELCOLOID O (propylene glycol alginate) have been determined. These data are tabulated below:

Table 4
Biological Oxygen Demand[1]

Product	5-Day	10-Day	20-Day	C.O.D.
KELGIN MV	285	394	408	665
SUPERLOID	334	469	499	720
KELCOLOID O	124	184	266	1,238

(1) Data reported as mg Oxygen/g Substrate

V Kelco Alginates

The alginic acid derivatives produced by Kelco vary in a great many ways. Available for use by processors are the sodium, potassium, ammonium, and calcium salts of alginic acid as well as the acid itself. Mixed salts such as sodium-calcium and ammonium-calcium are also produced. Kelco also manufactures propylene glycol ester of alginic acid, the only organic derivative made commercially.

The normal variables include:

1. Molecular weight (viscosity grade)
2. Calcium content
3. Particle form—granular, fibrous
4. Mesh size of the particles or fibers.

On the following pages are basic data for several types of Kelco alginates (Table 5) and a tabulation of physical properties of many of these products (Table 6 A-J). The viscosity/concentration relationships for several of the water-soluble alginates are shown in Figures 10 A-F. Table 6-K illustrates the typical nutritional values of Kelco alginates.

Table 5
Typical Physical Properties

	Alginic Acid	Refined Sodium Alginate	Specially Clarified Sodium Alginate	Ammonium Alginate	Propylene Glycol Alginate
Moisture Content	7%	13%	9%	13%	13% max.
Ash	2%	23%	23%	2%	10% max.
Color	White	Ivory	Cream	Tan	Cream
Specific Gravity	—	1.59	1.64	1.73	1.46
Bulk Density (lbs/cu ft)	—	54.62	43.38	56.62	33.71
Browning Temp., °C	160	150	130	140	155
Charring Temp., °C	250	340, 460	410	200	220
Ashing Temp., °C	450	480	570	320, 470	400
Ignition Temp., °C	*	*	*	*	*
Heat of Combustion (Cal/g)	2.80	2.50	2.44	3.04	4.44
As a 1% Solution (Dist. water)					
Heat of Solution (Cal/g Soln.)	0.090	0.080	0.115	0.045	0.090
Refractive Index (20°C)	—	1.3343	1.3342	1.3347	1.3343
pH	2.9	7.5	7.2	5.5	4.3
Surface Tension (Dynes/cm)	53	62	70	62	58
Freezing Point Depression, °C	0.010	0.035	0.020	0.060	0.030

*Spontaneous combustion did not occur in an air environment.

Table 6-A
Refined Sodium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELGIN® HV	Ivory	Granular	30	800	10,000	Neutral
KELGIN MV	Ivory	Granular	30	400	6,000	Neutral
KELGIN F	Ivory	Granular	80	300	4,000	Neutral
KELGIN LV	Ivory	Granular	40	60	500	Neutral
KELGIN XL	Ivory	Granular	40	30	160	Neutral
KELGIN RL	Ivory	Granular	40	10	30	Neutral
KELVIS®	Ivory	Granular	150	760	9,000	Neutral

Table 6-B
Industrial Sodium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELTEX®	Tan	Granular	20	800	10,000	Neutral
KELTEX P	Tan	Granular	80	765	9,000	Neutral
KELTEX S	Tan	Granular	20	1,300	—	Neutral

Table 6-C
Specially Clarified Low-Calcium Sodium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELCO-GEL® HV	Cream	Fibrous	80	400	3,500	Neutral
KELCO-GEL LV	Cream	Fibrous	150	50	250	Neutral
KELCOSOL®	Cream	Fibrous	80	1,300	15,000	Neutral
KELCO-PAC	Ivory	Granular	20	55	280	Neutral
KELTONE®	Cream	Fibrous	150	400	3,500	Neutral

Table 6-D
Refined Ammonium Alginate

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
SUPERLOID®	Tan	Granular	20	1,500	17,000	5.5

*These data were obtained using a Brookfield Model LVF Viscometer at 60 rpm with the appropriate spindle.

Table 6-E
Refined Potassium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELMAR®	Cream	Granular	100	270	3,200	Neutral
Improved KELMAR	Cream	Fibrous	80	400	4,500	Neutral

Table 6-F
Propylene Glycol Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELCOLOID® HVF	Cream	Fibrous	80	400	7,000	4.0
KELCOLOID DH	Cream	Agglomerated[1]	20	400	7,000	4.0
KELCOLOID D	Cream	Fibrous	40	170	2,000	4.4
KELCOLOID LVF	Cream	Fibrous	80	120	1,200	4.0
KELCOLOID O	Cream	Fibrous	80	25	130	4.3
KELCOLOID DO	Cream	Agglomerated[1]	20	25	130	4.3
KELCOLOID S	Cream	Fibrous	80	20	115	4.0
KELCOLOID DSF	Cream	Agglomerated[1]	60	20	115	4.0

[1]*Agglomerated for improved dispersion.*

Table 6-G
Sodium Alginates Treated for Improved Dispersion

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELGIN QH	Ivory	Granular	30	400	6,000	Neutral
KELGIN QM	Ivory	Granular	30	180	1,800	Neutral
KELGIN QL	Ivory	Granular	30	30	160	Neutral

Table 6-H
Miscellaneous Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELSET®[1]	Light Ivory	Fibrous	80	–	Soft Gel	Neutral
KELTOSE®	Ivory	Granular	80	–	Soft Gel	Neutral
MARGEL®	Cream	Granular	100	–	250	9.4

[1]self-gelling

**These data were obtained using a Brookfield Model LVF Viscometer at 60 rpm with the appropriate spindle.*

Table 6-I
Alginic Acid

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELACID®	White	Fibrous	80	Insoluble		3

Table 6-J
Dairy Stabilizers and Stabilizer/Emulsifiers (Milk Soluble)

Product	Color	Form	Approx. Mesh	pH
DARILOID®	Light Ivory	Granular	40	10.0
Concentrated DARILOID	Light Ivory	Granular	40	10.0
DARILOID K	Light Ivory	Granular	14	4.5
DARILOID KB	Light Ivory	Granular	14	5.4
DARILOID Q	Light Ivory	Granular	100	10.2
DARILOID QH	Light Ivory	Granular	100	10.2
DARILOID XL	Light Ivory	Granular	40	10.0
Concentrated DARILOID XL	Light Ivory	Granular	40	10.0
DRICOID® KB	Light Ivory	Granular	14	5.4

*These data were obtained using a Brookfield Model LVF Viscometer at 60 rpm with the appropriate spindle.

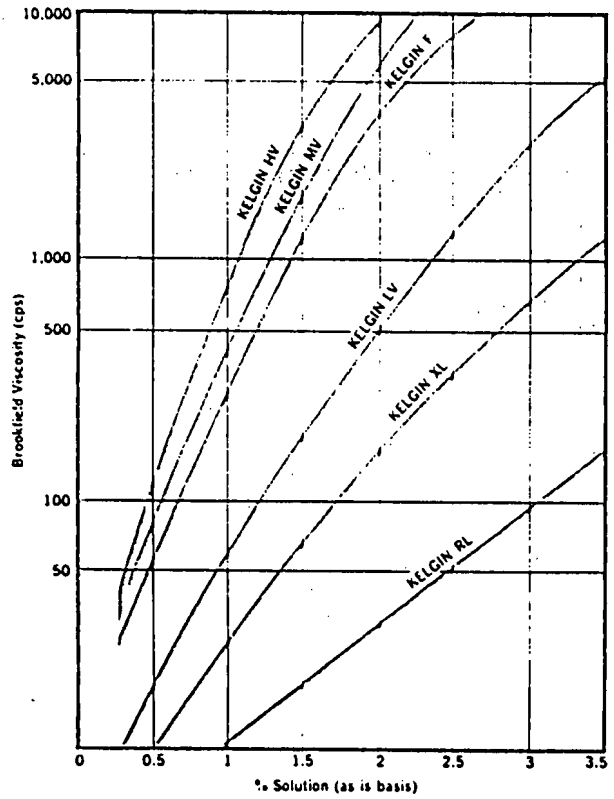
Table 6-K
Typical Nutritional Data of Kelco Alginates

Product	Cal.*	Carbo-hydrates (%)	Protein	Fat	Vitamins	Minerals (%) Na	Ca	Mg	K	P
COCOLOID®	2.2	77	Nil	Nil	Nil	7.4	0.6	0.03	0.30	1.1
Concentrated DARILOID	1.1	69	Nil	Nil	Nil	13.3	0.7	0.01	0.03	2.6
Concentrated DARILOID XL	1.1	69	Nil	Nil	Nil	13.3	0.7	0.01	0.03	2.6
Concentrated DARILOID KB	2.8	65	Nil	Nil	Nil	1.1	0.7	0.05	0.20	Nil
DARILOID	1.6	71	Nil	Nil	Nil	11.8	0.5	0.01	0.03	2.1
DARILOID K	2.4	76	Nil	Nil	Nil	1.7	0.6	0.01	0.30	Nil
DARILOID KB	3.3	86	Nil	Nil	Nil	0.5	0.2	0.01	0.10	Nil
DARILOID Q	2.2	77	Nil	Nil	Nil	7.3	0.4	0.01	0.01	1.9
DARILOID QH	2.2	77	Nil	Nil	Nil	7.3	0.5	0.01	0.02	1.9
DARILOID XL	1.5	71	Nil	Nil	Nil	11.1	0.5	0.01	0.02	1.9
DRICOID	3.8	47	Nil	26%	Nil	6.9	0.3	0.01	0.02	1.1
DRICOID KB	4.6	60	Nil	23%	Nil	0.5	0.2	0.01	0.10	Nil
SHERBELIZER	3.3	87	Nil	Nil	Nil	0.6	0.4	0.08	0.50	Nil
KELCOLOID D	2.4	70	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID DH	2.4	72	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID DO	2.5	70	Nil	Nil	Nil	1.2	0.3	0.01	0.02	0.1
KELCOLOID DSF	2.5	70	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID HVF	2.4	72	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID LVF	2.4	72	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID O	2.5	69	Nil	Nil	Nil	1.2	0.3	0.01	0.02	0.1
KELCOLOID S	2.5	69	Nil	Nil	Nil	1.2	0.3	0.01	0.02	0.1
KELTOSE	1.3	81	Nil	Nil	Nil	0.4	3.7	0.10	0.02	Nil
Improved KELMAR	1.2	76	Nil	Nil	Nil	0.4	0.3	0.01	9.40	Nil
KELACID	1.5	92	Nil	Nil	Nil	1.2	1.2	0.01	0.02	Nil
KELCO-PAC	1.3	79	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELCO-GEL HV	1.3	80	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELCO-GEL LV	1.3	80	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELCOSOL	1.3	80	Nil	Nil	Nil	7.7	1.0	0.01	0.01	Nil
KELMAR	1.2	75	Nil	Nil	Nil	0.4	1.2	0.01	9.40	Nil
KELSET	1.3	81	Nil	Nil	Nil	6.5	3.0	0.02	0.10	Nil
KELTONE	1.3	81	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELVIS	1.3	80	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
MARGEL	1.0	64	Nil	Nil	Nil	5.5	8.7	0.01	0.03	Nil
KELGIN F	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN HV	1.3	80	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN LV	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN MV	1.3	80	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN RL	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN XL	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil

*kilocalories per gram

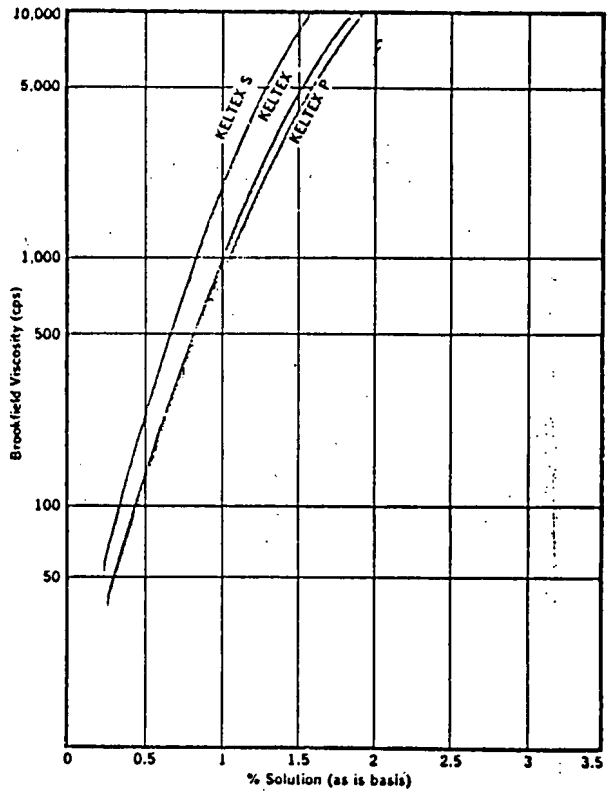
This information is supplied for material-labeling purposes. The analyses are representative and should not be construed as product specifications.

Figure 10-A. Viscosity/concentration curves for refined sodium alginates.



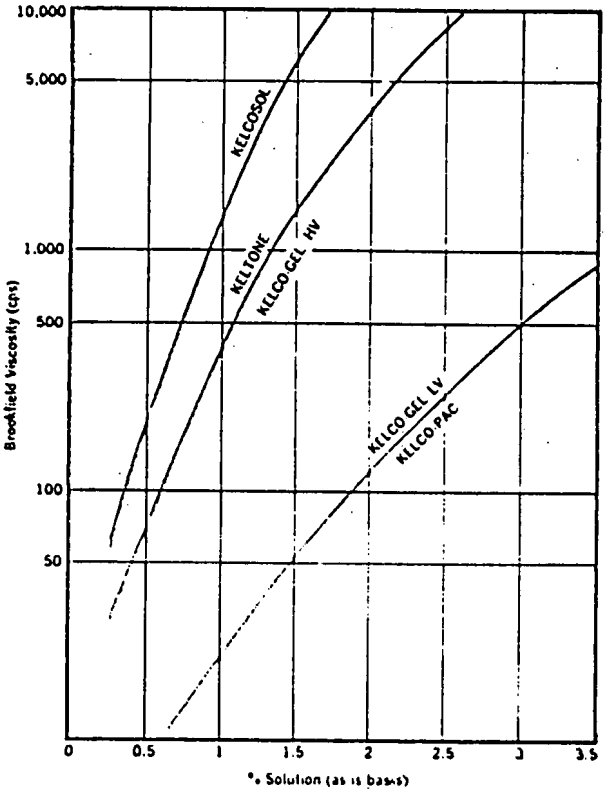
25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Figure 10-B. Viscosity/concentration curves for industrial sodium alginates.



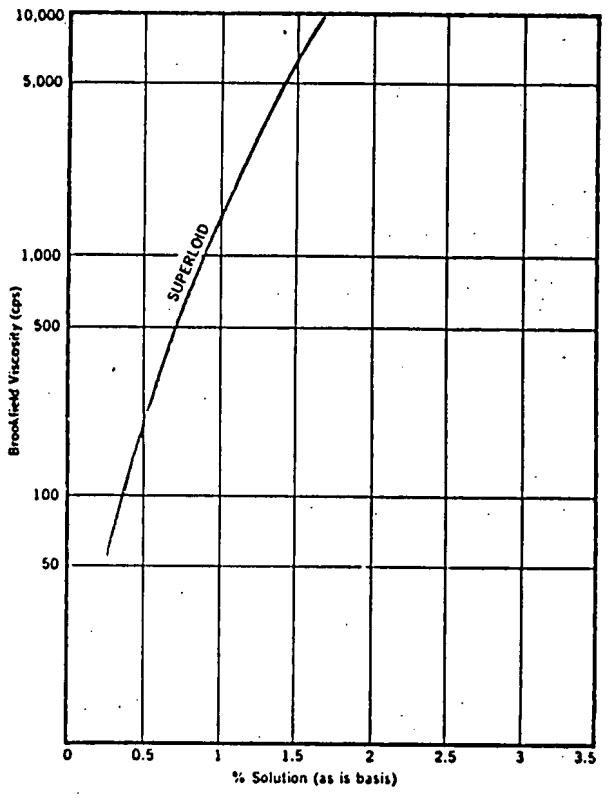
25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Figure 10-C. Viscosity/concentration curves for specially clarified sodium alginates.



25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Figure 10-D. Viscosity/concentration curves for refined ammonium alginate.



25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Table 7
Dry Powder Viscosity Loss
After 1 Year at 75°F

Product	Type	1% Solution Viscosity (cps)* Initial	After 1 Year
KELGIN MV	Sodium Alginate	420	380
KELGIN XL	Sodium Alginate	27	26
KELMAR	Potassium Alginate	270	248
KELTONE	Sodium Alginate	400	330
SUPERLOID	Ammonium Alginate	1,500	675
KELCOLOID HVF	Propylene Glycol Alginate	400	236
KELCOLOID LVF	Propylene Glycol Alginate	115	67

Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle.

General Comments

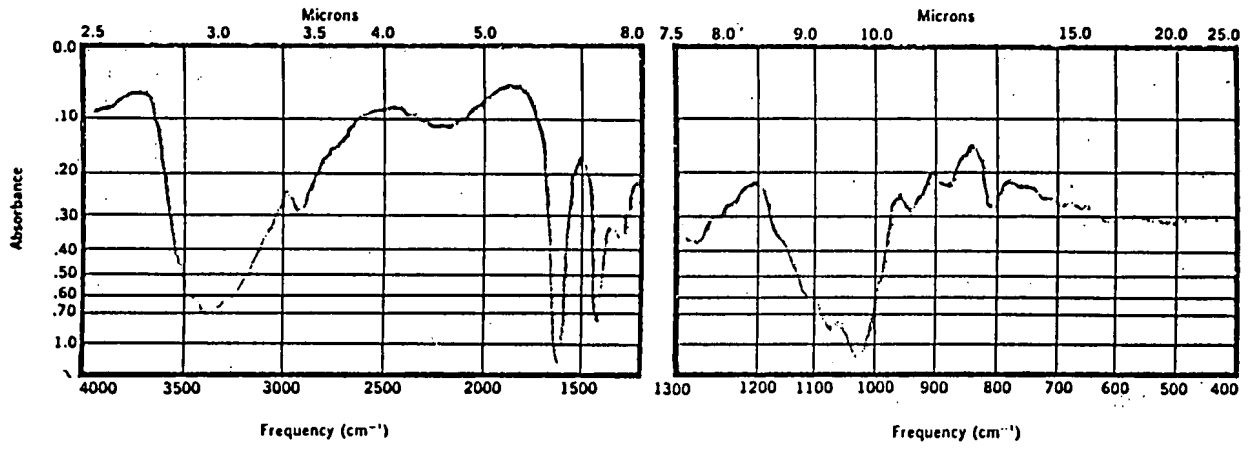
1. High-viscosity alginates usually decrease in viscosity faster than low-viscosity alginate.
2. Ammonium alginate is generally less stable than sodium, potassium, or propylene glycol alginates.
3. Propylene glycol alginates gradually become insoluble when stored at elevated temperatures for extended periods of time.

Table 8
Effect of Storage Temperature
On Viscosity After 1 Year

Product	Type	Storage Temp.	1% Solution Viscosity (cps)* Initial	After 1 Year
KELGIN MV	Sodium Alginate	35°F	420	410
		75°F		380
		90°F		230
KELGIN XL	Sodium Alginate	35°F	27	26
		75°F		26
		90°F		22
SUPERLOID	Ammonium Alginate	35°F	1,500	1,350
		75°F		625
		90°F		300

Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle.

Figure 14. Typical infrared spectrum of a purified sodium alginate obtained from a film.



VII Properties of Algin Solutions

When dissolved in distilled water, pure alginates form smooth solutions having long flow properties. The solution properties are dependent on both physical and chemical variables.

The physical variables which affect the flow characteristics of algin solutions include temperature, shear rate, polymer size, concentration in solution, and the presence of miscible solvents. The effects of shear rate, polymer size, and solution concentration on rheology are examined in detail in Section VIII, "Rheology of Algin Solutions." The effect of polymer size and concentration in solution can be seen in the viscosity/concentration curves in Section V, "Kelco Alginates."

The chemical variables affecting algin solutions include pH, sequestrants, monovalent salts, polyvalent cations, and quaternary ammonium compounds.

Table 9

Viscosity versus Time and Temperature Data in the Absence of Sodium Hexametaphosphate

Hours	77°F Room Temp.	100°F Hot	100°F Room Temp.	130°F Hot	130°F Room Temp.	160°F Hot	160°F Room Temp.	180°F Hot	180°F Room Temp.
			KELGIN HV						
			1% As Is Viscosities in Cps						
0	900	600	800	420	740	360	680	300	
1	900	540	720	400	660	310	600	220	510
2	900	520	700	380	640	290	570	200	480
3	900	500	680	360	620	270	550	180	460
4	900	490	670	350	610	260	530	170	440
			KELGIN MV						
			1% As Is Viscosities in Cps						
0	530	330	400	215	330	185	295	180	290
1	530	290	385	200	310	170	285	160	280
2	530	280	380	195	280	165	275	155	260
3	530	275	370	190	270	160	260	150	250
4	530	260	360	185	265	155	250	145	240
			KELGIN MV						
			1¼% As Is Viscosities in Cps						
0	1000	680	880	450	680	370	625	345	605
1	1000	610	830	410	650	320	600	300	580
2	1000	600	800	400	600	310	570	290	550
3	1000	590	780	390	580	300	550	285	530
4	1000	575	760	380	560	290	535	275	510
			KELGIN LV						
			1% As Is Viscosities in Cps						
0	62	40	54	26	51	22	44	19	40
1	62	38	54	25	49	22	43	19	40
2	62	37	52	25	47	21	42	19	39
3	62	37	52	24	47	20	41	18	38
4	62	36	51	24	46	20	40	18	37
			KELGIN XL						
			1% As Is Viscosities in Cps						
0	40	32	38	22	36	19	34	17	32
1	40	30	36	20	34	18	32	16	30
2	40	28	34	20	32	18	30	16	28
3	40	27	33	19	31	17	29	16	27
4	40	26	32	19	30	17	28	15	26

Physical Variables

A. Temperature

Algin solutions, like those of most other polysaccharides, decrease in viscosity with an increase in temperature. Over a limited range, the viscosity of an algin solution decreases approximately 12% for each ten degrees Fahrenheit increase in temperature. Tables 9 and 10 provide data for refined sodium alginates (KELGIN) of four molecular weights. Data were taken for solutions both without and with added sodium hexametaphosphate. Details on the laboratory procedure used in this evaluation follow in item "H" of this section. It is apparent that heating of sodium alginate solutions results in some thermal depolymerization and that the amount of depolymerization is dependent on both time and temperature.

Temperature reduction causes a viscosity increase in an algin solution but does not result in gel formation. A sodium alginate solution which has been frozen and then thawed will not have its appearance or viscosity changed. A freeze-dried sodium-calcium alginate gel can be formed which will have an absorptive capacity of over 5,000 percent but which will be water-disintegrative *(Wise 1972)*.

Table 10
Viscosity versus Time and Temperature Data in the Presence of Sodium Hexametaphosphate
(0.5 gram of Sodium Hexametaphosphate/gram of Algin)

Hours	77°F Room Temp.	100°F Hot	100°F Room Temp.	130°F Hot	130°F Room Temp.	160°F Hot	160°F Room Temp.	180°F Hot	180°F Room Temp.
				KELGIN HV					
				1% As Is Viscosities in Cps					
0	352	248	310	160	230	130	210	105	200
1	352	235	305	155	225	125	205	100	195
2	352	225	300	150	220	120	200	95	185
3	352	220	295	145	215	115	195	93	182
4	352	215	290	140	210	110	190	90	180
				KELGIN MV					
				1% As Is Viscosities in Cps					
0	155	120	145	65	100	55	100	50	90
1	155	115	130	65	95	55	95	48	86
2	155	108	125	58	90	50	90	44	82
3	155	105	120	55	90	46	85	40	80
4	155	100	115	50	85	42	85	38	76
				KELGIN MV					
				1¼% As Is Viscosities in Cps					
0	330	246	285	115	200	100	190	88	175
1	330	230	270	110	185	95	185	84	165
2	330	215	255	105	175	85	170	75	155
3	330	210	245	100	165	80	160	70	150
4	330	200	235	90	160	75	155	65	140
				KELGIN LV					
				1% As Is Viscosities in Cps					
0	31	22	30	18	29	15	27	13	25
1	31	22	29	18	28	15	26	13	24
2	31	21	29	17	27	14	25	13	23
3	31	21	28	17	26	14	24	12	22
4	31	20	27	16	26	13	23	12	21
				KELGIN XL					
				1% As Is Viscosities in Cps					
0	20	17	20	16	19	15	18	14	17
1	20	17	20	16	19	15	18	14	17
2	20	16	19	15	18	14	17	13	16
3	20	16	19	15	18	14	17	13	16
4	20	16	18	15	18	14	17	13	16

B. Water-Miscible Solvents

Alginates, as hydrophilic colloids, form aqueous solutions. The addition of increasing *amounts* of non-aqueous water-miscible solvents (alcohols, glycols, acetone, etc.) to an algin solution results in viscosity increases and eventual precipitation. The source of the algin, the degree of polymerization, the cation present, and the concentration in solution all affect the solvent tolerance of the algin solution.

A tabulation of quantities of solvent required for precipitation of algin is given in Table 11.

Chemical Variables

C. Effect of pH

The curves in Figure 15 illustrate the effect of pH variation on the viscosity of solutions of several types of alginates. Sodium alginates with some residual calcium content (KELGIN MV) gel at a pH of 5 and are unstable above a pH of about 11. Sodium alginates with minimal calcium content do not gel until the pH is 3 to 4 (KELCO-GEL LV).

It has been determined that lower-molecular-weight sodium alginates are stable at a pH as low as 3.0 if calcium is completely sequestered. Propylene glycol alginates do not gel until the pH is below 3 but do saponify above pH 6.7.

Although sodium alginate solutions appear to tolerate high pH conditions, long-term stability is poor above about pH 10. At higher pH, β elimination and hydrolysis result in depolymerization with an accompanying viscosity loss.

Figure 15. Effect of pH on algin solutions.

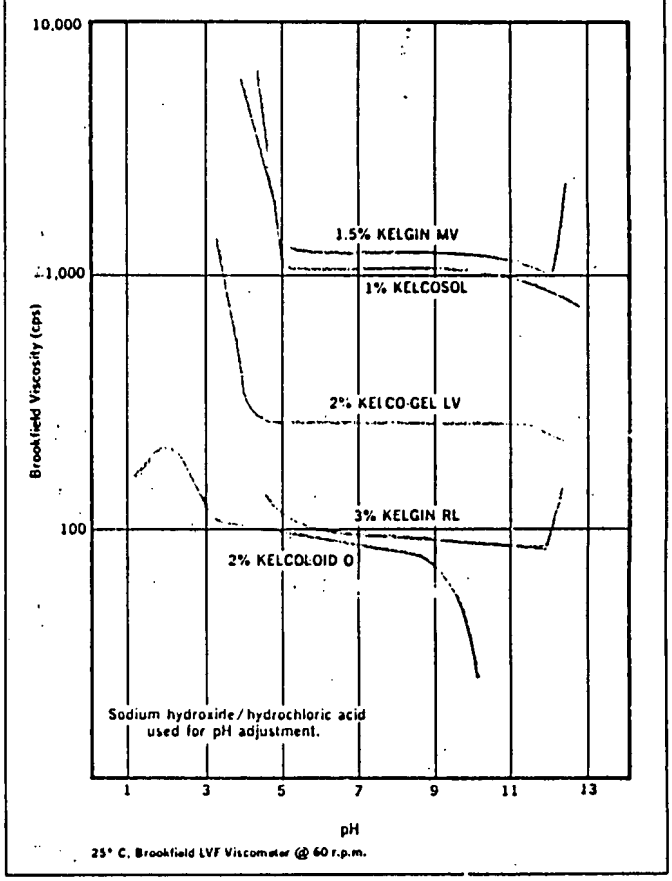


Table 11
Maximum Solvent Tolerance of Algin Solutions

	Methanol	Ethanol	Isopropanol	t-Butanol	Glycerol	Ethylene Glycol	Propylene Glycol	Butyl Cellosolve	Acetone
2% KELGIN XL	20%	20%	10%	20%	70%+	70%+	40%	20%	10%
1% KELGIN MV	20%	20%	10%	10%	70%+	70%+	40%	30%	10%
1% KELGIN HV	20%	20%	10%	20%	70%+	70%+	70%+	20%	10%
1% KELTONE	40%	40%	40%	40%	70%+	70%+	70%+	40%	20%
1% Improved KELMAR	20%	20%	20%	20%	70%+	70%+	50%	40%	20%
1% SUPERLOID	30%	30%	20%	20%	70%+	70%+	70%+	20%	20%
1% KELSET	40%	20%	20%	30%	60%	60%	50%	30%	30%
2% KELCOLOID O	20%	20%	20%	30%	70%+	70%+	50%	30%	10%
1% KELCOLOID D	20%	10%	10%	10%	70%+	70%	40%	40%	10%
1% KELCOLOID LVF	40%	30%	30%	30%	60%	70%+	50%	30%	30%
1% KELCOLOID HVF	30%	20%	20%	20%	60%	70%+	40%	30%	20%

Comments on data:

1. Algin was dissolved in water first, then diluted with solvent to a given water-solvent ratio. Concentration of algin in final solution was 1 percent (except 2 percent for KELGIN XL and KELCOLOID O). Solvent levels were varied in 10 percent increments.
2. The "maximum solvent tolerance" is the solvent percentage next below the percentage at which algin separation was evident.
3. With most solvents, the compatibility end point is quite evident due to algin precipitation and/or viscosity loss. With glycerol and ethylene glycol, however, the end point is not sharp, and apparent compatibility may extend to as high as 90 percent solvent. High solvent combinations are difficult to prepare because the algin must be hydrated prior to addition of the solvent.

Table 14
Algin Compatibility

Material	% Material	% KELGIN MV	Viscosity (cps)* Initial	Viscosity (cps)* After 90 Days
1. Preservatives				
Dowicide® A	0.005	1.0	395	265
	0.1	1.0	350	260
Formaldehyde	0.1	1.0	315	275
	1.0	1.0	325	310
Methyl Parasept®	0.5	1.0	345	285
Vancide® TH	0.1	1.0	275	207
	0.5	1.0	280	202
Sindar® G-4	0.1	1.0	285	222
	0.5	1.0	286	115
Advacide® 340-A	0.1	1.0	392	257
	0.5	1.0	390	268
Omacide® 24	0.1	1.0	375	420
	0.5	1.0	370	650
Surflo® B-17	0.1	1.0	377	320
	0.5	1.0	361	325
Nalco® 248	0.1	1.0	345	192
	0.5	1.0	295	195
Nalco 243	0.1	1.0	346	25
	0.5	1.0	322	227
Metasol® D3T	0.1	1.0	360	217
	0.5	1.0	347	147
Metasol DX3-S	0.1	1.0	332	167
	0.5	1.0	325	125
2. Thickeners				
Xanthan Gum	0.5	0.5	760	730
	1.0	1.0	3,740	4,000
Guar Gum	1.0	1.0	8,320	2,950
Gum Tragacanth	0.5	0.5	350	1,050
	1.0	1.0	5,170	9,150
Methocel® 90HG	0.5	0.5	290	160
Locust Bean Gum	0.5	0.5	340	220
	1.0	1.0	3,950	3,000
3. Water Soluble Resins				
Vinol® PA-20	1.0	1.0	550	350
	5.0	1.0	1,420	230
Carboset® 525	1.0	1.0	480	395
	10.0	1.0	370	290
Carbopol® 934	0.5	0.5	540	100
	1.0	0.5	1,250	650
4. Latex Emulsions				
Rhoplex® AC490	60.0	0.5	170	150
Ucar® 360	60.0	0.5	215	30
Dow Latex 460	60.0	0.5	330	300
Dow Latex 307	60.0	0.5	320	280
Geon® 652	60.0	0.5	280	10
Airflex® 500	60.0	0.5	470	260
Genflo® 355	60.0	0.5	380	405
Genflo 67	60.0	0.5	980	850
5. Organic Solvents				
Acetone	10.0	1.0	590	610
	20.0	1.0	1,710	3,150
Methanol	10.0	1.0	660	680
	20.0	1.0	1,590	3,000
Isopropanol	10.0	1.0	840	970
	20.0	1.0	2,720	4,250
Benzyl Alcohol	10.0	1.0	660	480

*Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle

Table 14 Continued

Material	% Material	% KELGIN MV	Viscosity (cps)* Initial	Viscosity (cps)* After 90 Days
6. Enzymes				
Alkalase*	1.0	1.0	190	155
Cellulase* 4000	1.0	1.0	315	195
Papain	1.0	1.0	323	223
Rhozyme* A-4	1.0	1.0	310	210
Gumase* HP 150	1.0	1.0	290	165
7. Surfactants				
Stepanol* WAT	10.0	0.5	43	37
Igepal* CO 630	20.0	0.5	569	250
Tween* NPX	20.0	0.5	1,290	630
Tergitol* NPX	20.0	0.5	340	220
Miranol* 2 MCA	10.0	0.5	28	38
8. Plasticizers (Glycols)				
Glycerol	50.0	0.5	1,240	3,750
Propylene Glycol	50.0	0.5	325	305
Triethanolamine	50.0	0.5	320	305
Hexylene Glycol	50.0	0.5	990	480
Kromfax*	50.0	0.5	1,010	1,450
Ethylene Glycol	50.0	0.5	1,000	1,120
9. Inorganic Salts				
Ammonium Chloride	1.0	1.0	250	1,100
	5.0	1.0	250	510
	10.0	1.0	230	145
Diammonium Phosphate	1.0	1.0	240	210
	5.0	1.0	210	150
	10.0	1.0	280	180
Ammonium Sulfate	1.0	1.0	235	150
	5.0	1.0	210	60
Magnesium Chloride	1.0	1.0	290	580
	5.0	1.0	130	120
Potassium Chloride	1.0	1.0	210	1,480
	5.0	1.0	210	350
Potassium Phosphate, Dibasic	1.0	1.0	190	170
	5.0	1.0	290	230
Potassium Sulfate	1.0	1.0	290	430
	5.0	1.0	290	230
Sodium Chloride	1.0	1.0	310	880
	5.0	1.0	340	1,090
Sodium Phosphate, Dibasic	1.0	1.0	230	160
	5.0	1.0	215	215
Sodium Sulfate	1.0	1.0	260	360
	5.0	1.0	265	255
Sodium Tetraborate	1.0	1.0	155	175
Sodium Citrate	1.0	1.0	215	170
	5.0	1.0	240	245

*Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle

XIII Applications of Kelco Algin Products

Kelco alginates have many applications in foods and industrial products due to their unique properties. The following table illustrates how some of the principal properties apply to typical products:

Food Applications

Property	Product	Performance
Water-holding	Frozen foods	Maintains texture during freeze-thaw cycle.
	Pastry fillings	Produces smooth, soft texture and body.
	Syrups	Suspends solids, controls pouring consistency.
	Bakery icings	Counteracts stickiness and cracking.
	Dry mixes	Quickly absorbs water or milk in reconstitution.
	Meringues	Stabilizes meringue bodies.
	Frozen desserts	Provides heat-shock protection, improved flavor release, and superior meltdown.
	Relish	Stabilizes brine, allowing uniform filling.
Gelling	Instant puddings	Produces firm pudding with excellent body and texture; better flavor release.
	Cooked puddings	Stabilizes pudding system, firms body, and reduces weeping.
	Chiffons	Provides tender gel body that stabilizes instant (cold make-up) chiffons.
	Pie and pastry fillings	Cold-water gel base for instant bakery jellies and instant lemon pie fillings. Develops soft gel body with broad temperature tolerance; improved flavor release.
	Dessert gels	Produces clear, firm, quick-setting gels with hot or cold water.
	Fabricated foods	Provides a unique binding system that gels rapidly under a wide range of conditions.
Emulsifying	Salad dressings	Emulsifies and stabilizes various types.
	Meat and flavor sauces	Emulsifies oil and suspends solids.
Stabilizing	Beer	Maintains beer foam under adverse conditions.
	Fruit juice	Stabilizes pulp in concentrates and finished drinks.
	Fountain syrups, toppings	Suspends solids, produces uniform body.
	Whipped toppings	Aids in developing overrun, stabilizes fat dispersion, and prevents freeze-thaw breakdown.
	Sauces and gravies	Thickens and stabilizes for a broad range of applications.
	Milkshakes	Controls overrun and provides smooth, creamy body.

Industrial Applications

Property	Product	Performance
Water-holding	Paper coating	Controls rheology of coatings; prevents dilatancy at high shear.
	Paper sizings	Improves surface properties, ink acceptance, and smoothness.
	Adhesives	Controls penetration to improve adhesion and application.
	Textile printing	Produces very fine line prints with good definition and excellent washout.
	Textile dyeing	Prevents migration of dyestuffs in pad dyeing operations. (Algin is also compatible with most fiber-reactive dyes).
Gelling	Air freshener gel	Firm, stable gels are produced from cold-water systems
	Explosives	Rubbery, elastic gels are formed by reaction with borates.
	Toys	Safe, nontoxic materials are made for impressions or putty-like compounds.
	Hydro-mulching	Holds mulch to inclined surfaces; promotes seed germination.
	Boiler compounds	Produces soft, voluminous flocs easily separated from boiler water.
Emulsifying	Polishes	Emulsifies oils and suspends solids.
	Antifoams	Emulsifies and stabilizes various types.
	Latices	Stabilizes latex emulsions, provides viscosity.
Stabilizing	Ceramics	Imparts plasticity and suspends solids.
	Welding rods	Improves extrusion characteristics and green strength.
	Cleaners	Suspends and stabilizes insoluble solids.

A comparative study of the cytotoxicity of silver-based dressings in monolayer cell, tissue explant, and animal models

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ABSTRACT

Over the past decade, a variety of advanced silver-based dressings have been developed. There are considerable variations in the structure, composition, and silver content of these new preparations. In the present study, we examined five commercially available silver-based dressings (Acticoat™, Aquacel® Ag, Contreet® Foam, PolyMem® Silver, Urgotul®SSD). We assessed their cytotoxicity in a monolayer cell culture, a tissue explant culture model, and a mouse excisional wound model. The results showed that Acticoat™, Aquacel® Ag, and Contreet® Foam, when pretreated with specific solutes, were likely to produce the most significant cytotoxic effects on both cultured keratinocytes and fibroblasts, while PolyMem® Silver and Urgotul®SSD demonstrated the least cytotoxicity. The cytotoxicity correlated with the silver released from the dressings as measured by silver concentration in the culture medium. In the tissue explant culture model, in which the epidermal cell proliferation was evaluated, all silver dressings resulted in a significant delay of reepithelialization. In the mouse excisional wound model, Acticoat™ and Contreet® Foam indicated a strong inhibition of wound reepithelialization on the postwounding-day 7. These findings may, in part, explain the clinical observations of delayed wound healing or inhibition of wound epithelialization after the use of certain topical silver dressings. Caution should be exercised in using silver-based dressings in clean superficial wounds such as donor sites and superficial burns and also when cultured cells are being applied to wounds.

The use of silver as an antimicrobial agent has a long history.[1] Its recent resurgence follows from Moyer's use of silver nitrate solution in patients with burns.[2] Solutions gave way to cream formulations but it is now silver-containing dressings that provide the widest range of silver-based wound care products.[3,4] Despite the ever increasing number of commercially available silver-based dressings, there is a distinct lack of comparative data on their clinical effectiveness.[5] What is known is that silver can be effective against a wide range of microorganisms, including aerobic, anaerobic, Gram-negative and Gram-positive bacteria, yeast, fungi, and viruses. The antimicrobial effect of silver can be explained by various mechanisms: silver interferes with the respiratory chain in the cytochromes of microbacteria; additionally, silver ions also interfere with components of the microbial electron transport system, bind DNA, and inhibit DNA replication.[6,7]

Dressings are designed to have more controlled and prolonged release of silver during the entire wear-time when compared with the cream formulations. This allows dressings to be changed less frequently, thereby reducing risk of nosocomial infection, cost of care, further tissue damage, and patient discomfort. Many factors affect the clinical performance of a dressing. The amount of silver content, the chemical, and physical forms of the silver, silver distribution, and even the affinity for moisture all participate in a dressing's capability to exert a significant antimicrobial effect. Marked differences exist in a variety of silver dressing products. These products can be categorized as: (i) silver-delivery dressings such as Acticoat™ (Smith & Nephew, Hull, UK) and Urgotul®SSD (Laboratory URGD, Chenove, France), which have silver content coated (on the surface) or impregnated into the dressing material. They deliver silver to the wound site after direct contact; (ii) silver-containing dressings such as Aquacel®Ag (Cenvatec, Deeside, UK), Contreet® Foam (Coloplast, Humlebaek, Denmark), and PolyMem® Silver (Ferris Mfg. Corp., Burr Ridge, IL), which have high absorptive capacities and "lock up" the silver content until the dressing absorbs wound exudate or moisture. In

DMEM	Dulbecco's modified Eagle media
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGM	Fibroblast growth medium
ICP-MS	Inductively coupled plasma mass spectrophotometer
KGM	Keratinocyte growth medium
K-SFM	Keratinocyte serum-free medium
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
PWD	Postwounding day

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Exhibit M

Urgotul® SSD, the silver is in the form of silver sulfadiazine, which is released into the wound bed. This is metabolized to release silver and sulfadiazine moeties.[8] Traditionally, the active silver agent has been thought to be ionic silver but in the nanocrystalline form, elemental silver is also thought to be active.[9] Independent studies involving comparisons between different types of dressings suggested considerable variations in one or more aspects of dressings' performance.[4,9–11] Our published microbiology data demonstrated that Acticoat™ and Contreet® Foam have a broad spectrum of bactericidal activities against both Gram-positive and -negative bacteria, and Contreet® Foam was characterized by a very rapid bactericidal action.[12] However, we and others have also demonstrated in vitro cytotoxic effects of silver nitrate ($AgNO_3$), silver sulfadiazine, and Acticoat™ on cultures of keratinocytes and fibroblasts.[13–15]

In the present study, we are concerned with the cytotoxicity of a range of commercially available silver dressings to the viable cells in the wound bed. We have examined the biological effects of these dressings on: first, isolated skin cells—the monolayer culture of human keratinocytes and fibroblasts; second, the tissue explant culture model—pig mid-dermis culture for epidermal cell proliferation; and last, the mouse excisional wound model.

MATERIALS AND METHODS

Materials

Five silver-based dressings (Acticoat™, Aquacel® Ag, Contreet® Foam, PolyMem® Silver, Urgotul®SSD) and one control dressing without silver (Aquacel®) were obtained from commercial sources. The components of these dressings are summarized in Table 1.

Human keratinocyte and fibroblast cultures were developed from primary cultures of discarded surgical tissues or foreskins, according to Institution Ethical guidelines. 3T3/NIH fibroblasts were obtained from the American Type Culture Collection (ATCC; Rockville, MD). All the media and reagents used in cell cultures were purchased from GIBCO (Grand Island, NY) unless specified.

Six- to 8-month-old large white pigs and C57 BL/6J mice were sourced from the Laboratory Animal Services Centre of the Chinese University of Hong Kong (CUHK). All animal procedures were subject to the approval of the Animal Experimentation Ethics Committee of the CUHK, and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Keratinocyte culture

Keratinocytes were isolated from the skin tissues of discarded surgical tissues or foreskins as previously described.[15] Briefly, surgical samples were collected in skin transport medium comprising Dulbecco's modified Eagle media (DMEM) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL Fungizone® (Invitrogen, Grand Island, NY), and 50 µg/mL gentamicin. The skin biopsy was transferred to the laboratory and trimmed by a surgical blade to remove all adipose tissue and cut into 2–3 mm wide strips before immersion in 2 mg/mL dispase II (Roche Diagnostics,

Table 1. Dressings

Dressing Name	Manufacturer	Basic dressing composition	Silver composition	Silver release
AQUACEL®	ConvaTec (Deeside, UK)	Hydrocolloid fiber (sodium carboxymethylcellulose)	None	—
AQUACEL® Ag	ConvaTec	Hydrocolloid fiber (sodium carboxymethylcellulose)	1.2% w/w ionic silver (silver nitrate)	Ag^+
Acticoat™	Smith & Nephew (Hull, UK)	An absorbent polyester inner core sandwiched between two outer layers of silver-coated polyethylene net	Metallic nanocrystalline silver	Ag^0
Contreet® Foam	Coloplast (Humlebaek, Denmark)	Polyurethane foam	Ionic silver (silver sodium hydrogen zirconium phosphate)	Ag^+
PolyMem® Silver	Ferris Mfg. Corp. (Burr Ridge, IL)	Polyurethane foam containing a safe nontoxic cleanser (F-68 surfactant), a moisturizer (glycerol) and an absorbing agent (superabsorbent starch copolymer)	Elemental nanocrystalline silver (124 ug/cm^2)	Ag^0
Urgotul® S.S.D	Laboratory URGO (Chenove, France)	Polyester gauze dressing impregnated with hydrocolloid particles dispersed in a Vaseline paste	Silver sulfadiazine	Ag^+

Basel, Switzerland) in DMEM for overnight digestion at 4 °C. The following day, the epidermis was mechanically separated from the dermis and collected into 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA) solution. Basal keratinocyte cells were dissociated by vortex for 1 minute and neutralized with three volumes of DMEM with 10% FBS. The cells were then pellected by spinning at 200×*g* for 5 minutes. Cells were resuspended in keratinocyte growth medium (KGM) consisting of one volume of Ham's F12, three volumes of DMEM, 10% FBS, 100 U/mL penicillin, 100 μL/mL streptomycin, 0.25 μg/μL amphotericin B, 100 fM cholera enterotoxin, 5 μg/mL transferring, 180 μM adenine sulfate, 5 μg/mL insulin, 10 mg/mL epidermal growth factor, 0.4 μg/mL hydrocortisone (Sigma), and 20 fM liothyronine (Sigma). The cell suspension was seeded onto 4 μg/mL mitomycin C-treated-3T3/NIH feeder layer at a density of $3\times10^4/cm^2$. Keratinocytes were subcultured at 70–80% confluence after differential dissociation of the feeder layer with 0.02% EDTA for 10 minutes, followed by keratinocyte cell dispersion with 0.05% trypsin-0.02% EDTA treatment for 5 minutes. Cells were expanded one more passage on feeder cells and stored under liquid nitrogen in KGM with 5% dimethyl sulfoxide (DMSO) and 20% FBS. Cells at passages 3–5 were used in the following experimental assays.

Fibroblast culture

The dermis was separated from the skin tissues of discarded surgical tissues or foreskins by dispase digestion as previously described.[15] The dermis was then finely minced, and resultant cell suspension, together with small pieces of tissues, were transferred to culture dishes and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μL/mL streptomycin, and 0.25 μg/μL amphotericin B (fibroblast growth medium, FGM). Medium was changed weekly. Upon reaching confluence, cells were passaged using 0.25% trypsin-EDTA. Cells at passages 4–10 were used in the following experimental assays.

Dressing absorbency test

The dressing absorbency test was performed according to the instructions from the "Test methods for primary wound dressings-Part 1: Aspects of absorbency" issued by the State Food and Drugs Administration, People's Republic of China, which is compatible with the European reference of Test methods for primary wound dressings—the EN 13726:2002 – section 3.2 free-swelling capacity. In brief, the testing dressing was cut into $1\times1\ cm^2$ size and was weighed (W_0). A solution A composed of 142 mmol Na ions and 2.5 mmol Ca ions was first prewarmed to 37 °C, and then the testing dressing as well as solution A were placed onto a 100 mm Petri dish and incubated at 37 °C for 30 minutes. The volume of solution A required was 40 times larger than the weight of the dressing. The dressing was weighed again after a 30-minute incubation (W_{30}). The absorbency of the dressing was expressed as volume of solution A absorbed per square centimeter dressing ($[W_{30}–W_0]$/area) and set as one of the parameters for dressing pretreatment as described below.

Pretreatment of dressing materials

All dressings were cut into $1\times1\ cm^2$ size under a sterile condition. Based on the dressing absorbency test result, PolyMem® Silver was found to have the highest absorbency of 0.8 mL/cm^2. The time for the foam dressings to be fully saturated with solution A was 10 minutes. Therefore, for the pretreatment, all the dressings were soaked with 0.8 mL of different solutes—deionized water, saline, and 100% FBS, respectively, for 10 minutes at 37 °C. The dressings, as well as individual pretreatment solutions, were then added to the culture for cytotoxicity assay.

Cytotoxicity testing of dressing materials on keratinocyte and fibroblast monolayer cultures

The cytotoxicity testing of six types of dressings on both keratinocyte and fibroblast monolayer cultures was performed in the same manner, except for using different culture medium and cell densities. Briefly, keratinocytes were seeded into six-well plates at a density of 1×10^5/well and cultured in defined keratinocyte serum-free medium (K-SFM; GIBCO)-containing insulin, epidermal growth factor, and fibroblast growth factor. Fibroblasts were seeded at a density of 5×10^4/well in FGM. Upon 3–4 days culture until 70–80% cell confluence, the $1\times1\ cm^2$ dressings were presoaked with 0.8 mL of deionized water, saline, or FBS, respectively, as mentioned above. Afterward, the dressings together with individual pretreatment solutions, and 2.2 mL of culture medium, were added to each of the culture wells. Addition of 0.8 mL of the plain solute without dressing being soaked was regarded as a positive control. The cells were then incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 2, 4, 6, and 24 hours. At each time point, dressings were removed and cell viability was determined by an 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Experiments were performed in triplicate.

MTT assay

MTT is a yellow dye that is taken up by viable cells and converted to Formazen 8 (purple crystals). Change in color can be assessed spectrophotometrically to give an assessment of metabolic activity as a function of cytotoxicity.[15] At each time point of the cytotoxicity testing, dressings from each well were removed and culture medium was aspirated. One milliliter MTT solution (0.5 mg/mL) in complete culture medium was added to each cell-containing well and then incubated in a humidified atmosphere for 2–4 hours in the dark. Afterward, 1 mL solubilization solution (10% SDS in 0.01 M HCl) was added and the plates were maintained at 37 °C in an incubator in a humidified atmosphere overnight. Afterward, 200 μL of the solubilization solution from each well was transferred to a 96-well enzyme-linked immunosorbent assay (ELISA) plate for spectrophotometric measurement using a microplate (ELISA) reader (Spectra Rainbow, TECAN, Grödig, Austria), with a test wavelength of 570 nm and a reference wavelength of 690 nm.

The results of the MTT assay were expressed as the relative cell viability of individual experimental treatment to that of the control group at 2 hours (mean ± SD). The

statistical significance was assessed by Student's t-test using Prism 3.0 software from Graphpad, and $p < 0.05$ was considered to be significant.

Silver content of dressing materials

Samples of each dressing in $1 \times 1\,cm^2$ were digested by heating in a mixture of concentrated HNO_3 and HCl to break down the dressing matrix and to release and dissolve all of the silver present. The digest was then filtered and diluted with deionized water. Total silver in the aqueous samples was determined by inductively coupled plasma mass spectrometry (ICP-MS, 7,500c, Agilent Technologies Inc., Palo Alto, CA). Sample aerosol generated by a neubilizer was carried to an argon plasma of about 8,000 K for the production of silver ions. The sample ion was then introduced into a mass spectrometer for ion identification and quantification. For verification of the accuracy of the analyte, a standard reference material (SRM 1,577b, Bovine Liver, the National Institute of Standards and Technology, Gaithersburg, MD) was analyzed along with the samples. The sensitivity of the assay was 1.6 nmol/L. The interassay coefficients of variations were 3.7% at 38 nmol/L and 2.5% at 320 nmol/L. The total extractable silver content of each dressing was then determined and expressed in $\mu g/cm^2$.

Silver dissociation of dressing materials in different solution

To determine silver dissociation of each dressing in different solutions, dressing samples in $1 \times 1\,cm^2$ piece were presoaked with 0.8 mL of deionized water, saline or 100% FBS, respectively, for 10 minutes and forwarded to 2.2 mL culture medium, K-SFM, or FGM, respectively, for incubations at 37 °C for 2, 4, 6, and 24 hours. The extraction solution was then centrifuged at 940g for 10 minutes at 4 °C, and 200 µL of each supernatant was collected for ICP-MS assay as described above. The total amount of silver released over time was then determined and expressed in microgram per liter (µg/mL).

Pig mid-dermis explant culture

This model is based on a previously described technique.[16] Briefly, mid-dermal sheets from the paravertebral areas of 6–8-month-old large white pigs were harvested under aseptic conditions using a Pagett's dermatome at a setting of 0.5 mm after removing a 0.5 mm thick split-thickness graft containing the epidermis. Dermal strips were cut into $1 \times 1\,cm^2$ segments and transferred onto sterile dressing gauze in Petri dishes to raise the explant to the air–liquid interface for culture. Orientation of the dermis was maintained. Explants were maintained in 5% CO_2 environment with 95% humidity in serum-free DMEM supplemented by 10 ng/mL hydrocortisone. The testing dressings of size $1 \times 1\,cm^2$ each were laid on the surface of the explants on day 0. The cultures were harvested at days 4 and 8, and subject to formalin fixation and rhodamine staining for visualization of the reepithelialized areas around the hair follicles. Resurfaced areas were photographed and their size was determined using computerized morphometric analysis (Metamorph 4.0). The ratio of area of reepithelialization to the number of hair follicles in a certain area was determined to give an index of reepithelialization.

Mouse excisional wound model

This model is based on that described by Galiano et al.[17] Four full-thickness excisional wounds extending through the panniculus carnosus were created on each C57 BL/6J mouse using a 6-mm diameter biopsy punch. A silicone splint was added onto each wound and sutured to reduce the contraction effect during wound closure. The wounds were either dressed with testing dressings or not dressed (control), followed by a cover of Tegaderm (a semi-occlusive dressing) to hold the dressings in place. At two time-points, post-wounding day 4 (PWD 4) and 7 (PWD 7), the whole-wound tissues were dissected and subjected to standard histological examination of the reepithelialization. The epithelial gap (EG) and wound gap (WG) of each wound were measured at microscopic level using image analysis software. The percentage of EG/WG (EGW) for each wound was then calculated as an indicator of wound-healing rate.

RESULTS

In vitro cytotoxicity of silver-based dressings on cultured human keratinocyte

As shown in Figure 1, the relative cell viability of keratinocytes after different dressing treatments varied significantly.

In control treatments in which three types of plain solute without dressing being soaked was individually added (see "Materials and Methods"), addition of serum (FBS) but not water and saline markedly suppressed the proliferation of keratinocyte. Addition of 0.8 mL of FBS in total volume of 3 mL culture medium led to a 30% reduction of cell viability at 24 hours when compared with that at 2 hours.

Aquacel®, as a control dressing without silver, showed mild cytotoxicity on keratinocytes when delivered by water and saline. About 88 and 75% viable keratinocytes remained, respectively, after 24 hours dressing delivery by water and saline, respectively. When delivered in FBS, no additional reduction of cell viability was obviously observed compared with the control treatment.

Acticoat™ exerted a severe cytotoxic effect on keratinocytes when delivered by water. Eighty percent of cells were killed immediately after the dressing was delivered with water for 2 hours. After 24 hours only $1.6 \pm 0.33\%$ viable cells remained. However, by using saline as a pretreatment solute, cell viability was significantly improved and remained around 80% during 24 hours. On the other hand, FBS pretreatment also improved cell viability but still led to 50% cell death after 24 hours.

Aquacel® Ag was cytotoxic to keratinocytes in all three pretreatment statuses. The relative cell viability at 24 hours was $53.1 \pm 5.04\%$, $9.0 \pm 5.80\%$, and $19.9 \pm 11.53\%$, respectively, when the dressing was delivered by water, saline, and FBS pretreatment, respectively.

Contreet® Foam was severely cytotoxic to keratinocytes when delivered by FBS. More than 95% cells were killed immediately after the dressing was delivered with FBS for 2 hours and the killing effect lasted till 24 hours. Using water and saline as the pretreatment solutes, this

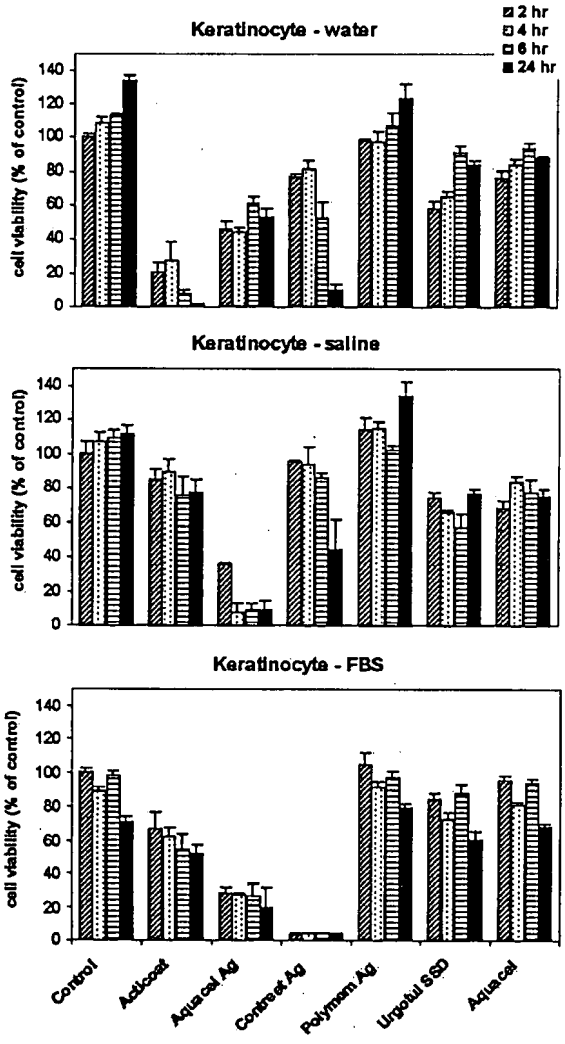

Figure 1. The relative cell viability of keratinocytes after various dressing treatments for 2, 4, 6, and 24 hours was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dressings were pretreated with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). Experiments were performed in triplicate. The results are expressed as percentage of the cell viability of individual experimental treatment to that of the control group at 2 hours (mean ± SD).

dressing did not promote a significant cell death within a short period but led to 90 and 50% cell death, respectively, at 24 hours.

PolyMem® Silver was relatively safe for keratinocytes in all three pretreatment statuses. No obvious cytotoxic effect was observed in comparison with individual controls. In fact, the dressing was shown to enhance keratinocyte proliferation slightly when delivered by saline.

Urgotul®SSD was also relatively safe for keratinocytes. Its biological activity on the growth of keratinocyte was comparable with that of the nonsilver-containing dressing, Aquacel®.

In brief, PolyMem® Silver was shown to be relatively safe for cultured keratinocytes, while Acticoat™, Aquacel® Ag, and Contreet® Foam were significantly lethal when delivered by specific pretreatment solute.

In vitro cytotoxicity of silver-based dressings on cultured human fibroblast

As shown in Figure 2, the response of cultured fibroblasts to different dressing treatments also varied significantly.

In control treatments, the growth of fibroblasts was not significantly affected by the addition of the plain pretreatment solutes, water, and saline. While fibroblasts responded differently from keratinocytes to the addition of serum solute (FBS): no cell growth inhibition, rather a doubled increase of fibroblast proliferation, was observed within 24 hours.

Aquacel®, as a nonsilver-containing dressing, hardly affected the cell growth of the fibroblast no matter which pretreatment solute was used. Within 24 hours of dressing delivery, fibroblasts proliferated similarly as they did in the control treatments.

Acticoat™ exerted a significant cytotoxic effect on fibroblasts when delivered by water. Seventy percent of cells were killed immediately after the dressing was delivered with water for 2 hours, and after 24 hours, only $25.2 \pm 0.78\%$ viable cells remained. By using saline as a pretreatment solute, the cell viability of fibroblast was improved but still remained less than 50% at 24 hours. However, the cell viability was improved to more than 70% at 24 hours when the dressing was delivered with FBS.

Aquacel® Ag exerted severe cytotoxicity on fibroblasts no matter which pretreatment solute was used. The lethal effect was observed immediately after 2 hours of dressing delivery. Less than 25% fibroblasts could survive after being exposed to Aquacel® Ag for 24 hours.

Contreet® Foam was also severely cytotoxic to fibroblasts after 24-hour delivery. Among the three pretreatment solutes, a relatively slow onset of action was observed when the dressing was delivered by saline pretreatment, $63.7 \pm 1.87\%$ cells remained after 2 hours, while 60.1 ± 5.17, 46.7 ± 8.36, and $22.9 \pm 3.31\%$ viable cells remained after 4, 6, and 24 hours, respectively.

PolyMem® Silver was observed to show mild cytotoxicity to fibroblasts within a short period. At 6 hours of the dressing delivery, about 77.5 ± 0.00, 63.1 ± 3.18, and $82.8 \pm 1.21\%$ viable cells remained, respectively, in water-, saline-, or FBS-delivered treatments. After 24 hours, the cells seemed tolerant to all the treatments and started to proliferate again.

Urgotul®SSD affected fibroblast in a manner similar to PolyMem® Silver. Using saline as the pretreatment solute, around 30% cells were killed within 6 hours of the dressing delivery, while the cells were observed to proliferate again at 24 hours.

In brief, PolyMem® Silver and Urgotul®SSD were the least cytotoxic to cultured fibroblasts, while Acticoat™, Aquacel® Ag, and Contreet® Foam showed significant killing or inhibitory effect on the cell growth of the fibroblast.

Silver content and silver dissociation of the dressings

The measured total silver content of the dressings is shown in Table 2, which indicates large differences between the

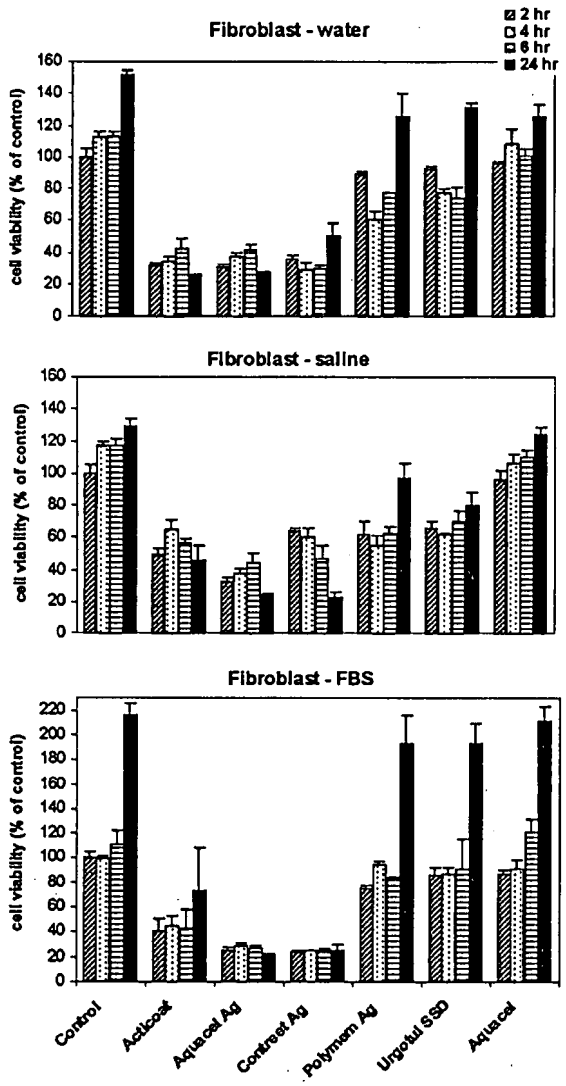

Figure 2. The relative cell viability of *Fibroblasts* after various dressing treatments for 2, 4, 6, and 24 hours was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dressings were pretreated with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). Experiments were performed in triplicate. The results are expressed as percentage of the cell viability of individual experimental treatment to that of the control group at 2 hours (mean ± SD).

five products, ranging from 13 μg for Contreet® Foam to 934 μg for Acticoat™ for a 1 cm^2 sample.

The amount of silver released into different culture medium including keratinocyte culture medium (K-SFM serum-free medium) and fibroblast growth medium (FGM—DMEM+10% FBS) after the dressing was presoaked with different solutes including water, saline, or FBS, also varied extensively over time. An increase in silver release was seen with the increase of the time. It is also noteworthy that, when the dressings were presoaked with

Table 2. Silver content of the dressings

	Ag content (μg/cm^2)
Acticoat™	934
Aquacel® Ag	21
Contreet® Foam	13
PolyMem® Silver	139
Urgotul®SSD	85

FBS, the amount of silver released into either keratinocyte culture medium or fibroblast growth medium was significantly improved, in particular, in the case of Acticoat™, Aquacel® Ag, and Contreet® Foam (Figures 3 and 4).

Comparison of the silver release and silver content for different dressings revealed no correlation. Comparison of the in vitro cytotoxicity and silver dissociation in different pretreatment solutes and culture mediums revealed that, generally, there was an obvious correlation of these two factors for different dressings (Figures 3 and 4). For example, when compared with Acticoat™, Aquacel® Ag, and Contreet® Foam, PolyMem® Silver, and Urgotul®SSD, two silver-containing dressings that showed the least cytotoxicity to both cultured keratinocytes and fibroblasts, released much less amount of silver into the culture medium over time. However, such a correlation was not always exactly consistent for highly cytotoxic dressings such as Acticoat™, Aquacel® Ag, and Contreet® Foam. For example, in keratinocyte cultures, when presoaked with saline, Contreet® Foam showed the largest silver release at 24 hours (Figure 3D). Aquacel® Ag, however, not Contreet® Foam, was found to be the most cytotoxic under the same conditions (Figure 1).

In situ cytotoxic effect of silver-based dressings on epidermal reepithelialization

The cytotoxic effects of silver-based dressings on an epidermal cell proliferation model—pig mid-dermis explant culture—were also examined. As shown in Figure 5A, in the control cultures, the reepithelialization index (reepithelialization area [mm^2] per hair follicle) kept growing from days 0 to 8, while a delayed reepithelialization was observed in all explant cultures covered by silver-based dressings. The least number and area of rhodamine-stained reepithelialized surface was seen in the Acticoat™-dressed explant culture, whose reepithelialization index was 0.083 ± 0.005 and 0.089 ± 0.014, respectively, at days 4 and 8. Histological examination (Figure 5B) also confirmed the significant reepithelialization in the control group, but not in samples treated with silver-based dressings.

In vivo cytotoxicity effects of silver-based dressings on wound reepithelialization

We used a mouse excisional wound model to study the effect of silver-based dressings on wound epidermal reepithelialization. As shown in Figure 6A, at the microscopic

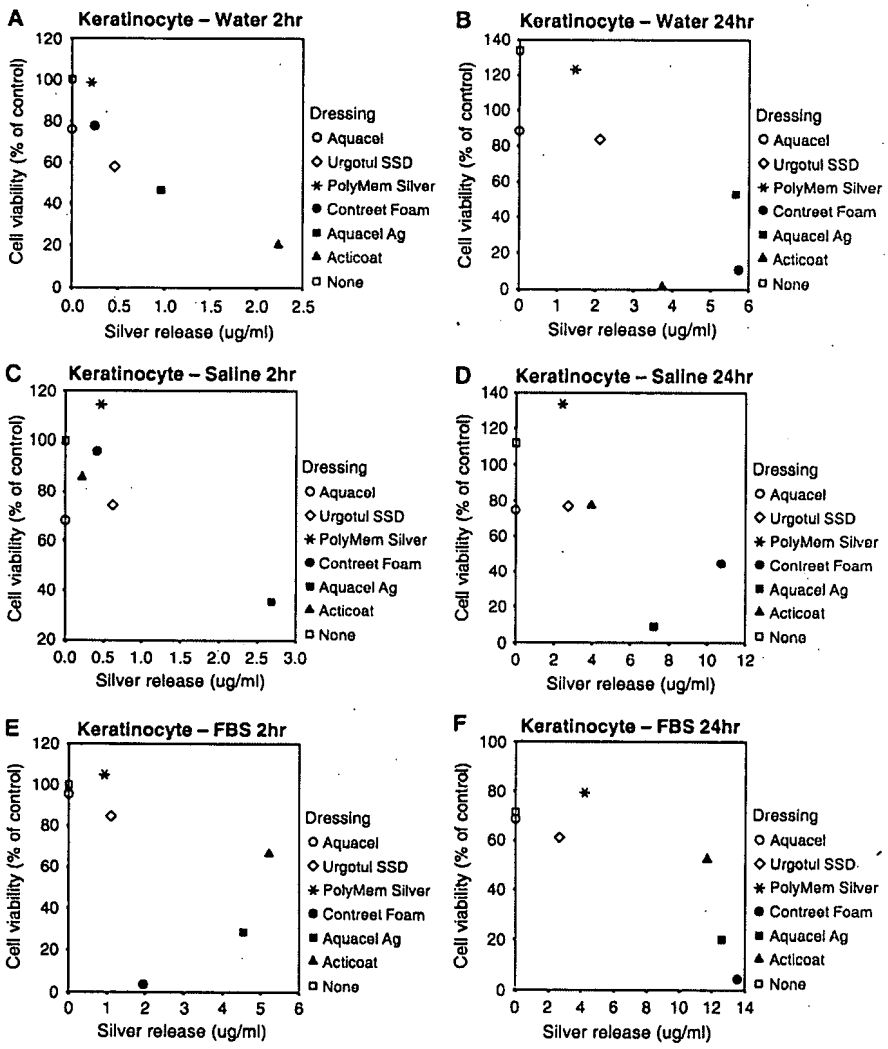

Figure 3. Comparison of the amount of silver released (ug/mL) into keratinocyte culture medium with the relative cell viability (%) after various dressing treatments for 2 and 24 hours. The dressings were presoaked with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). The total amount of silver released into the culture medium over time was determined by an inductively coupled plasma mass spectrophotometer (ICP-MS) assay. A scatter diagram was plotted to analyze the relationship between the silver concentration and the cell viability.

level, the epithelial tongue (ET) comprised keratinocytes growing from the adjacent unwounded epidermis toward the center of the wound. The epithelial gap (EG) and wound gap (WG) of each wound were measured; the percentage of EG/WG (EGW) for each wound was then calculated as an indicator of wound-healing rate (the larger the EGW, the more the delay in reepithelialization). The data are summarized in Figure 6B. As shown, the inhibitory effect of silver-based dressings was not significant on PWD4. On PWD7, Acticoat™, and Contreet® Foam indicated a strong inhibition of reepithelialization, the EGW of which were 71.4 and 73.1%, respectively; in comparison, the EGW of the control group was 25.9% only on PWD7. Aquacel® Ag, and PolyMem® Silver also impaired wound reepithelialization on PWD.[7]

DISCUSSION

The results from this study serve to underline the complex differences between a small range of commercially available dressings. At the outset, we sought to establish a "battery" of laboratory-based models which could be used to assess the potential clinical performance of the ever-rapidly proliferating range of silver-based dressings. Cell culture techniques are well established and highly reproducible and have been used by others and ourselves to assess silver toxicity.[15,18] There are limitations, however, to using monolayer cultures eloquently described by Lansdown as "naked cells" in a review of silver in wound care.[19] In our own clinical experience, we can acknowledge the value of hypochlorite solution in wound care which fell out of favor in the "Eusol" debate.[15] Thus, in this evaluation, we sought to extend the models and add to the biological complexity by using a three-dimensional tissue explant model and an animal model, albeit murine, of dynamic epidermal cell proliferation. It is also relevant to observe that in our clinical practice, the major focus is on healing wounds where infection is more a feature to be prevented, than treated, and unimpeded cell proliferation is the biological means to the clinical end point of wound closure. Having said that, the antimicrobial effect of the silver

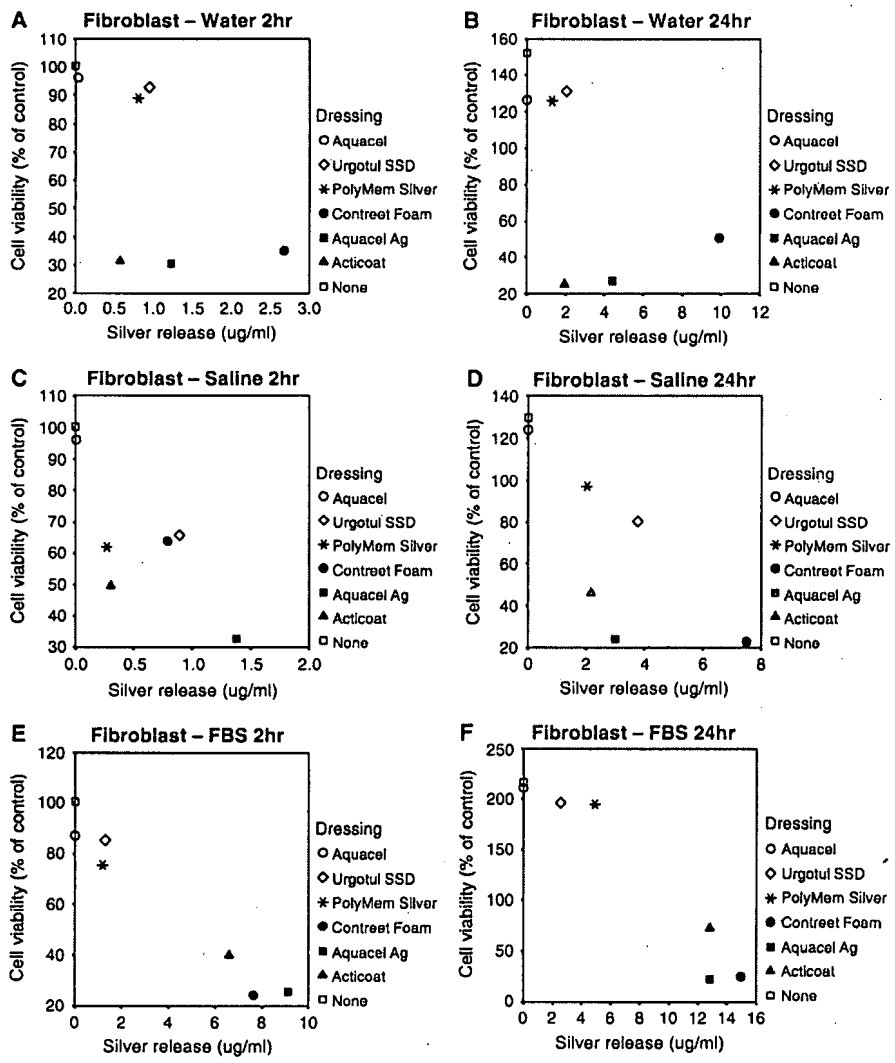

Figure 4. Comparison of the amount of silver released (ug/mL) into fibroblast culture medium with the relative cell viability (%) after various dressing treatments for 2 and 24 hours. The dressings were presoaked with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). The total amount of silver released into the culture medium over time was determined by an inductively coupled plasma mass spectrophotometer (ICP-MS) assay. A scatter diagram was plotted to analyze the relationship between the silver concentration and the cell viability.

based dressings remains a significant feature in our clinical application. It was anticipated that the ability of a dressing to exert a significant antimicrobial effect would be directly related to the silver content of the dressing and this was certainly demonstrated in one reported study.[7] The situation appears to be far less clear in the cytotoxicity studies. Thus, for example the measured silver content of PolyMem® Silver was 139 μg/cm^2, which is tenfold higher than that of Contreet® Foam (13 μg/cm^2). Yet, PolyMem® Silver had a less apparent cytotoxic effect than Contreet® Foam. More critical determinants of the potential cytotoxicity of a dressing are the nature of the dressing, in particular, its affinity for moisture as well as the silver composition, that is to say, the distribution of the silver within or on the dressing and the chemical and physical form of the silver (metallic, bound, or ionic). In this study, there are two preparations of nanocrystalline silver: Acticoat™ which delivers the silver from the surface, and, PolyMem® Silver, which is a foam-based dressing. We have demonstrated that PolyMem® Silver has the highest absorbancy among the dressings tested in this study. It also has less silver released into the carrier medium and thus it appears to be "locking up" the silver in the dressing. This is potentially a very good feature of a silver-based dressing where the bacterial "kill zone" is in the dressing rather than in the wound, thus avoiding the "collateral" damage to the healthy cells within the wound.

The test of absorbancy that we have used is based on that described by the State Food and Drugs Administration, China, which in turn is based on / compatible with European reference tests. We have scaled down the amount of material used for cost reasons but in all other respects have found this does give a true and representative comparison with published data. It should be emphasized that we are not aiming to prove or disprove the claims of any commercially sponsored research but rather to look for fair, reasonable, and independent models to evaluate the comparative performance of present and future dressings. The correspondence regarding the validity of testing methodologies is insightful in this regard with the 20-second absorption time in Parson's Convatec-sponsored paper[20] being criticized in Anderson's response (on

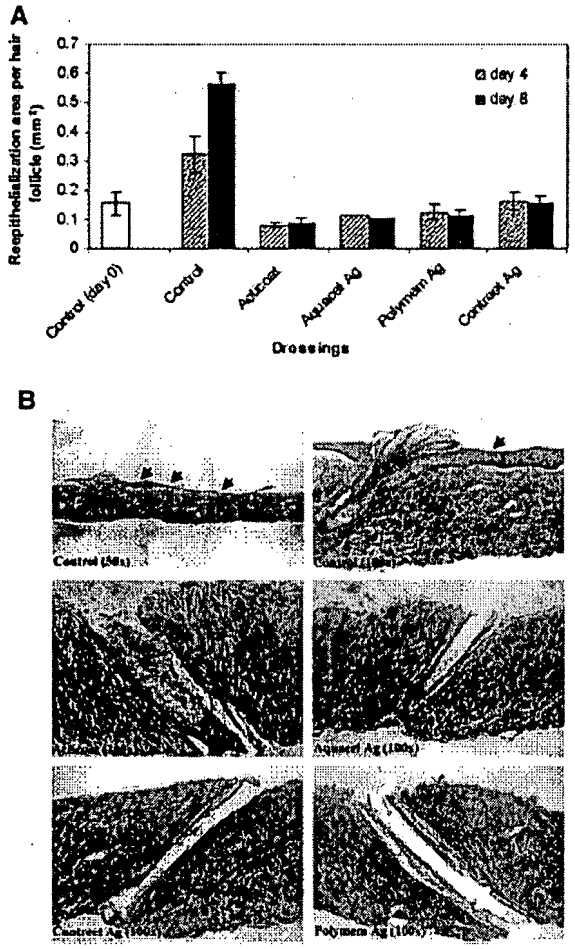

Figure 5. Silver dressings delay epidermal reepithelialization in pig mid-dermis explant culture. (A) The reepithelialization index (reepithelialization area [mm^2] per hair follicle) on days 4 and 8 postdressing delivery was determined (n=3). (B) Examples of histological examination (H&E staining) of cultured tissue explants on day 8 for different experimental treatment groups. The arrows represent reepithelialization areas.

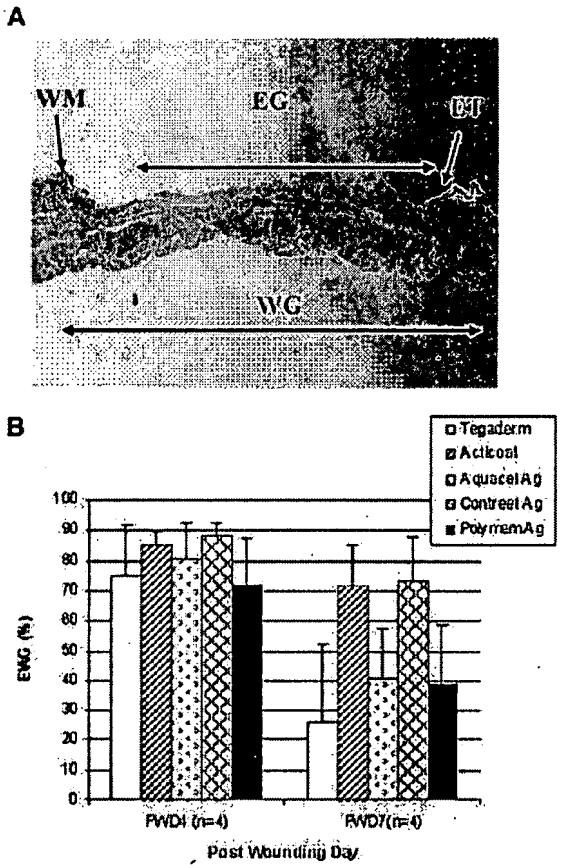

Figure 6. Silver dressings inhibit wound reepithelialization in a mouse excisional wound model. (A) An example of the histological examination (H&E staining) of wound section on postwounding day 4. WM, wound margin; ET, epithelial tongue; EG, epithelial gap; WG, wound gap. (B) Quantitative analysis of wound reepithelialization on postwounding days 4 and 7 for different experimental treatment groups. Data are expressed as the percentage of EG/WG (i.e., EWG %).

behalf of Coloplast).[21] It is also interesting that in the ensuing correspondence, the validity of methodology to determine silver release has also been challenged.

The decision to use a "pretreatment" solution was based on the clinical application of the dressing. According to the manufacturer's instruction, Acticoat™ needs to be moistened with deionzed water before use. Saline is expressly not to be used. Other dressings may be applied dry or moistened. The interaction with the wound will depend upon the fluid environment. For silver to be in a biologically active form, it must be soluble either as Ag^+ or Ag° clusters. Ag° is the metallic or unchanged form of silver found in the nanocrystalline formulations.[9]

What is perhaps surprising is the effect of the pretreatment solution on the cytotoxicity of the various dressings. As an example, Acticoat™ was found to have a significant cytotoxic effect on both keratinocytes and fibroblasts when pretreated with water. Pretreatment with saline significantly reduced the cytotoxicity. The release of silver from Acticoat™ pretreated in saline was significantly reduced when compared with Acticoat™ pretreated with water. The variation in the measured amount of silver released is again surprising. It must be appreciated, however, that the model system is far more complex than that used when manufacturers measure silver release. Thus, the data provided from a Coloplast-sponsored study of in vitro release profiles used a diffusion cell with a continuous flow of 1.4 mL/hour of release media consisting of an isotonic solution with equal amounts of sodium and serum.[22] In our system, the dressings were pretreated and then allowed to equilibrate in two different types of media in which the two cell types were grown. These media are both complex protein-containing solutions.

It was found in the present study that under all test conditions, Aquacel® Ag and Contreet® Foam released a comparable or even greater amount of silver ions than

Acticoat™ at 24-hours postdressing delivery. This, to some extent, was found to lead to a greater cytotoxic effect than Acticoat™. It is also noteworthy that when the dressings were presoaked with FBS, the amount of silver released into both keratinocyte culture medium (K-SFM) or fibroblast growth medium was significantly increased, in particular, in the case of Acticoat™, Aquacel® Ag, and Contreet® Foam. Serum is an extremely complex mixture of plasma proteins, growth factors, hormones, etc. The mechanism involved in serum-promoted silver release is yet unknown, but clearly and generally, the presence of sodium and chloride ions has an effect on silver dissociation.

The pig mid-dermis model is an explant culture model. It provides a three-dimensional system that can also be analyzed in four dimensions (time being the fourth dimension). We have previously used this model to assess the stimulation of cell proliferation and migration by the exogenous application of topical agents.[16] The use of a highly reproducible biological model was appealing and could also provide statistically appropriate comparative data when looking at the area of reepithelialization. In this study we have also compared vertical sections of the explants and selected those that showed clear sections of the hair follicle shaft. We have not elaborated a scoring system for this aspect and so the comparison becomes descriptive rather than statistical. Nevertheless, it is interesting to note that in all four dressings applied, the reepithelialization was not noted. As with other aspects of this current study, there is the potential to explore the biological mechanisms in more detail for example using immunohistochemical staining of matrix protein. This is, however, beyond the scope of this paper. The mouse wound-healing model again focuses on reepithelialization. The typical wound would quickly heal by contraction so the silicone splint keeps it open to allow closure by reepithelialization to be measured. Both models demonstrated delayed or inhibited reepithelialization by silver-based dressings. Taken together, our findings may explain the clinical observation of delayed wound healing or inhibition of wound epithelialization after the use of topical antimicrobial dressings. We suggest that silver-based dressings should be used with caution in situations where rapidly proliferating cells may be harmed as in donor sites, superficial burns, and application of cultured cells. It must be observed that previous studies that demonstrate the silver enhances acute wound healing were performed on incisional wounds where keratinocyte proliferation is not a major feature.[23]

Silver dressings are used in a wide range of wound-healing situations. In our clinical practice, we are more involved in the acute wounds and the emphasis is more on the prevention of infection and promotion of healing. It was the study of the clinical data pertaining to Acticoat™ and reepithelialization that caused our initial questioning of the validity of such studies. On the one hand, Innes et al. demonstrated that nanocrystalline silver impedes reepithelialization in donor sites,[24] while Demling and DeSanti were demonstrating that it increases the rate of reepithelialization in meshed skin grafts.[25] Is one or another of these studies flawed or is there an explanation for both to be correct?

We also have to recognize the clinical anomalies of positive cultures being grown from wounds that have been dressed with silver-based products. This cannot simply be attributed to silver resistance, which is in fact quite rare.[26] Rather, it may well be that the bacteria in vivo behave differently from the bacteria in vitro such that silver is not such an effective killer in the wound as it is in the laboratory.

The requirements of a dressing in a chronic wound-healing situation are different where the control of the wound bioburden is more important.[27] Of course, this was one of the strengths of the hypochlorite solutions that ran into disrepute because of their laboratory-based toxicity.[15] It is a concern when undertaking a study of commercially available materials that results will be quoted out of context. It is certainly not our intention to endorse or criticize any specific dressings. Rather, it has been our intention to explore the variability of the performance in the model systems described and thereby gain a greater understanding of the potential biological interactions of silver and wounds. It is important to keep in vitro derived data in perspective but this applies not only to the cell cytotoxity of silver products but also their ability to effectively deliver silver and kill bacteria in vivo. Ultimately, the best evidence to support clinical effectiveness will come from randomized prospective blinded studies. Such comparative studies are awaited.

ACKNOWLEDGMENT

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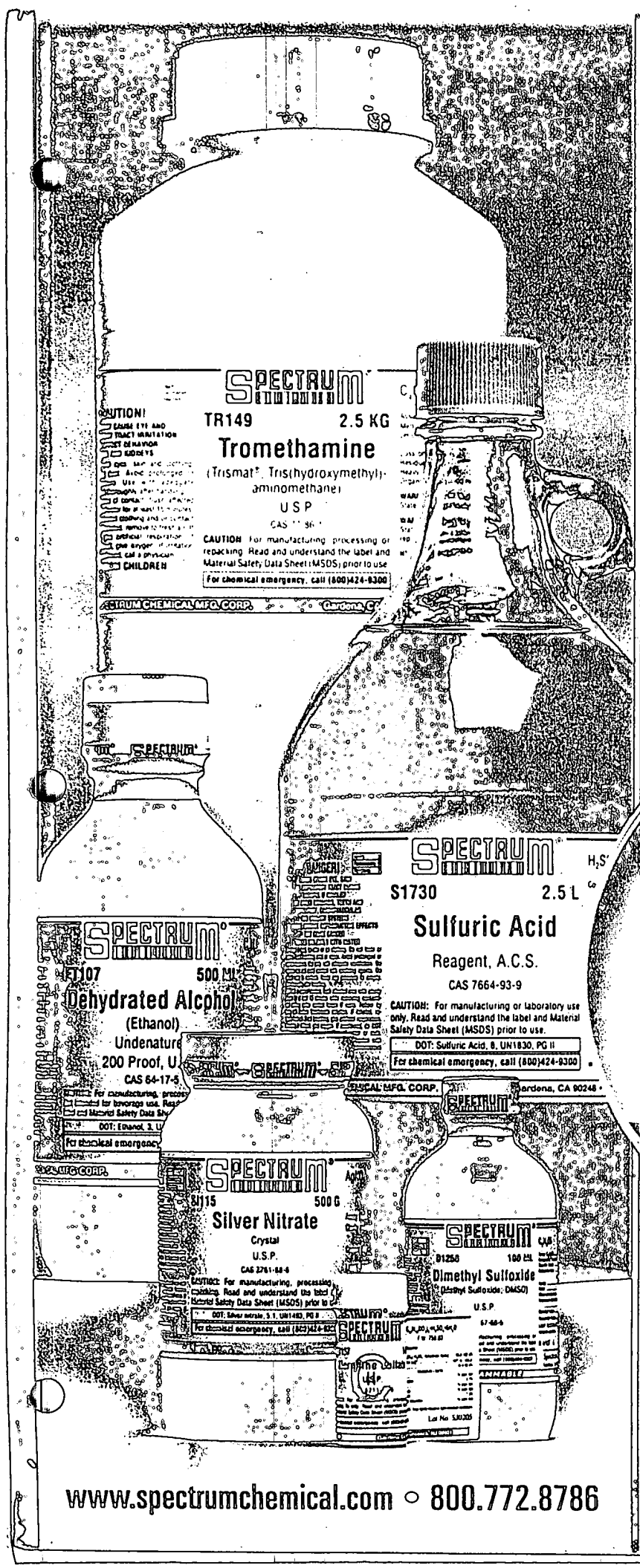

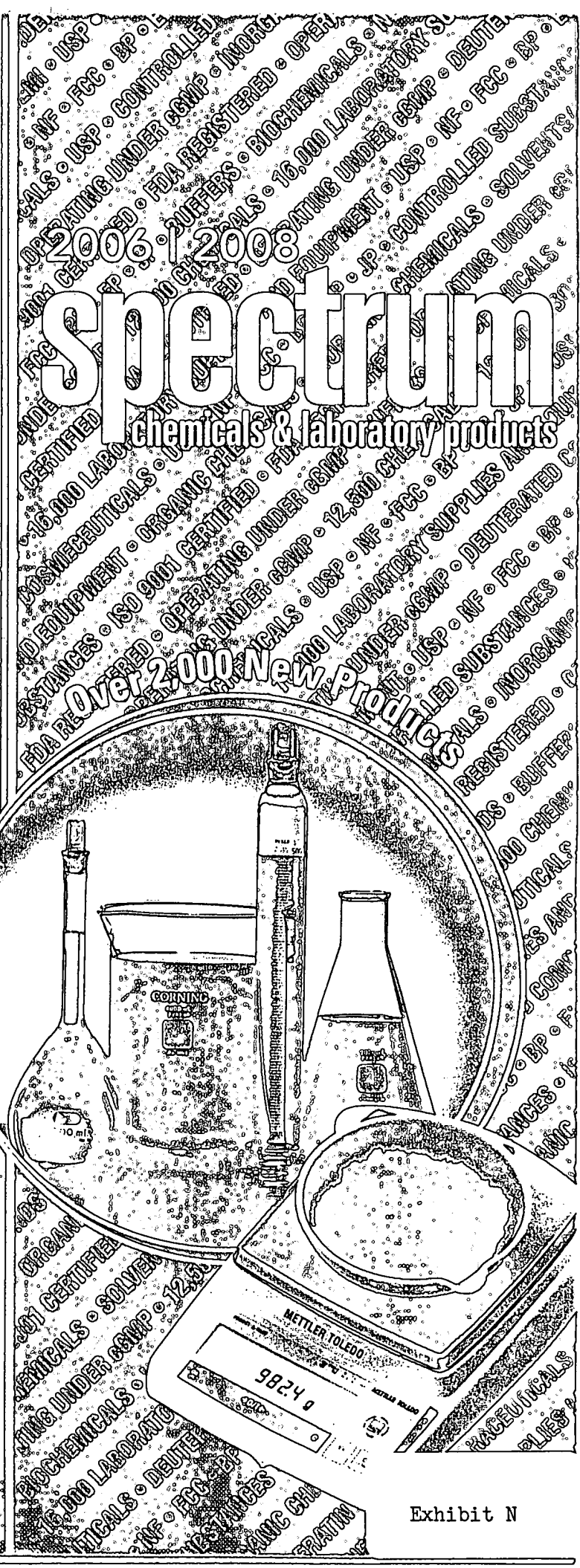

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Exhibit N

AG107

Agar, Flake, NF

CAS 9002-18-0

125 g	29.50
500 g	86.55
2.5 kg	355.65

Botanic Characteristics	As required

MAXIMUM LIMITS

Microbial Limit	To pass test
Water	20.0%
Total Ash (Dried Basis)	6.5%
Acid-Insoluble Ash (Dried Basis)	0.5%
Foreign Organic Matter	1.0%
Foreign Insoluble Matter	1.0%
Arsenic (As)	3 ppm
Lead (Pb)	0.001%
Heavy Metals	0.004%
Foreign Starch	To pass test
Gelatin	To pass test
Water Absorption	To pass test
Organic Volatile Impurities	To pass test

AG105

Agar, Granular, NF

CAS 9002-18-0

125 g	34.05
500 g	97.95
2.5 kg	376.85

Specifications: Same as AG107, p. 27

AG110

Agar, Powder, NF

Agar is the dried, hydrophilic, collodial substance extracted from *Gelidium cartilagineum* (Linne) Gaillon (Fam. *Gelidiaceae*), *Gracilaria confervoides* (Linne) Greville (Fam. *Sphaerococcaceae*), and related red algae (*Class Rhodophyaceae*).

CAS 9002-18-0

125 g	29.50
500 g	86.55
2.5 kg	355.65
25 kg	See Bulk Catalog

Specifications: Same as AG107, p. 27

1. Additional carrier-imposed hazardous material charge.

A1672
Agar, Powder, FCC
A dried hydrophilic, colloidal polygalactoside extracted from *Gelidium cartilagineum* (L.) Gaillon (Fam. *Gelidiaceae*), *Gracilaria confervoides* (L.) Greville (Fam. *Sphaerococcaceae*), and related red algae (Class *Rhodophyceae*).
CAS 9002-18-0

125 g 29.50
500 g 86.55
2.5 kg 355.65

MAXIMUM LIMITS

Arsenic (as As)	3 mg/kg
Ash (Acid-Insoluble)	0.5%
Ash (Total)	6.5%
Gelatin	To pass test
Insoluble Matter	1.0%
Lead (Pb)	5 mg/kg
Loss on Drying	20.0%
Starch	To pass test
Water Absorption	To pass test

A3953
Agaric Acid
[Agaricic Acid; 2-Hydroxy-1,2,3-nonadecanetricarboxylic Acid]
Has been used as antiperspirant.
$C_{22}H_{40}O_7$ F.W. 416.55 CAS 666-99-9

250 mg 60.80
1 g 152.00
5 g 505.00

STORE IN A COOL PLACE

A3572
Agarose, -0.13 EEO, Ultrapure
[Amresco Agarose I*]
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

10 g 46.55
25 g 94.85

Appearance	Off-white powder

MAXIMUM LIMITS

Loss on Drying	10%

A3627
Agarose, -0.17 EEO, Ultrapure
[Amresco Agarose I*]
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 204.40

Gelling Point (2.0%)	36°C
EEO	-0.17
Sulfate (SO_4)	0.35%

A1059
Agarose, High Gel Temperature, Electrophoresis Grade
[Amresco Agarose I*]
Applications: Nucleic acid electrophoresis; other procedures where very low electroendosmosis is desired.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 92.40
100 g 285.65

Appearance	To pass test
Gel Temperature (1.5%)	36°-39°C

MAXIMUM LIMITS

EEO	0.1
Sulfate (SO_4)	0.30%
Ash	0.5%
Moisture	10.0%
DNase, RNase and Protease	None detected

AG115
Agarose, Low Electroendosmosis, Electrophoresis Grade
[Amresco Agarose I*]
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 81.10
100 g 250.20

Gel Point	Approx. 36°C
Gel Strength, 1% @ 36°C	Min. 800 g/cm²

MAXIMUM LIMITS

EEO	0.15
Sulfate (SO_4)	0.35%

A3633
Agarose, Low Electroendosmosis, Low Sulfate
[Amresco Agarose I*]
Suitable for use in high resolution electrophoresis of serum plasma, cerebrospinal fluid and urine.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 54.55
100 g 187.50

MAXIMUM LIMITS

Sulfate (SO_4)	0.20%
Water (KF)	7.0%

A3638
Agarose, Medium Electroendosmosis
[Amresco Agarose I*]
Gelling agent for electrophoresis of macromolecules.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 75.50
100 g 228.95

Melting Point	Approx. 260°C

MAXIMUM LIMITS

Sulfate (SO_4)	0.25%

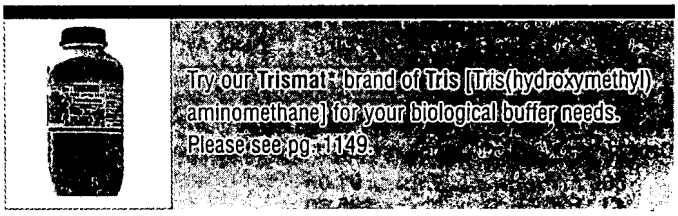
A3168
Agarose, Superfine Resolution, Electrophoresis Grade
[Amresco Agarose I*]
Same ability as acrylamide to resolve DNA fragments less than 1,000 bp. Suitable for the analysis of AMPFLP's, STR's and transnucleotide repeats.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 177.85
100 g 438.60
250 g 1060.20

Gel Strength (1.5%)	Min. 860 g/cm²
Melting Range (1.5%)	65°-69°C
pH of a 1% Solution in Water @ 25°C	As reported

MAXIMUM LIMITS

Gelling Temperature (1.5%)	30°C
EEO (-mr)	0.13
Sulfate (SO_4)	0.075%
DNase, RNase and Protease	None detected



*Trademark. See trademark index for ownership.

A3165

Agarose, High Resolution, Electrophoresis Grade

[Amresco Agarose I*]

Provides high resolution of small nucleic acid fragments and PCR* products.

$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 118.50
100 g 355.55
250 g 725.65

Appearance	Fine, white powder
Gel Strength (1.5%)	Min. 2,000 g/cm²
Gelling Range (1.5%)	34°-38°C
Melting Range (1.5%)	85°-89°C
MAXIMUM LIMITS	
EEO	0.12
Sulfate (SO_4)	0.10%
DNase, RNase and Protease	None detected
Function Test	To pass test
Endonuclease / Ligase Inhibitory Factors	None

A3171

Agarose, Type I, Electrophoresis Grade

[Amresco Agarose I*]

Suitable for a wide variety of nucleic acid and protein analytical applications.

$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 75.65
100 g 286.30
250 g 602.10

Gel Strength	Min. 1200 g/cm²
Gelling Range	36°-39°C
Melting Range	87°-89°C
MAXIMUM LIMITS	
EEO	0.10
Sulfate (SO_4)	0.15%
DNase, RNase and Protease	None detected

2120 and 2125

Agarose, OmniPure*

For Molecular Biology

Suitable for a wide range of nucleic acid and protein gel applications. Of average gel strength and standard melting and gelling ranges.

$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 342.80
500 g 1167.55

Appearance	Fine, white powder
Gel Strength (1.0%)	1200 g/cm²
Gelling Range (1.0%)	36°-39°C
Melting Point (1.0%)	87°-89°C
MAXIMUM LIMITS	
EEO (-mr)	0.10
Sulfate (SO_4)	0.15%
DNase, RNase and Protease	None detected

2090

Agarose, High Gel Strength, OmniPure*

For Molecular Biology

For pulsed field applications. Can also be used for resolving nucleic acids down to 200 bp.

$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 242.65

Appearance	White powder
Gel Strength (1.5%)	3200 g/cm²
Gelling Range (1.5%)	36°-37.5°C
Melting Point (1.5%)	87°-89°C
MAXIMUM LIMITS	
Ash	0.25%
EEO (-mr)	0.12
Sulfate (SO_4)	0.12%
Moisture	7.0%
DNase, RNase and Protease	None detected

2070

Agarose, Low Melting, OmniPure*

For Molecular Biology

Preparative agarose that melts at 65°C and remains liquid at 37°C for several hours.

$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 668.35

Appearance	White powder
Gel Strength (1.0%)	250 g/cm²
Gelling Range (1.0%)	27°-29.5°C
Melting Point (1.0%)	62°-68°C
MAXIMUM LIMITS	
EEO (-mr)	0.15
Sulfate (SO_4)	0.15%
DNase, RNase and Protease	None detected

2010

Agarose, PCR Plus, OmniPure*

For Molecular Biology

Especially for resolution of small DNA fragments of less than 1000 bp and PCR products. Prevents smearing or high fluorescence backgrounds.

$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 342.80
500 g 1167.55

Appearance	Fine, white powder
Gel Strength (1.5%)	2000 g/cm²
Gelling Range (1.5%)	34°-38°C
Melting Point (1.5%)	85°-89°C
MAXIMUM LIMITS	
EEO (-mr)	0.12
Sulfate (SO_4)	0.10%
Endonuclease/Ligase Inhibitory Factors	None detected
DNase, RNase and Protease	None detected

AGGC See N-Acetyl-S-geranylgeranyl-L-cysteine, p. 161

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OCT 25 2007
PATENT & TRADEMARK OFFICE

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October 22, 2007

Re: Application No: 09/818,928
Filing Date: March 28, 2001
Inventor: George H. Scherr
Title: Cellulose Foam Compositions
Examiner: Lezah W. Roberts

Lezah Roberts
United States Patent and Trademark Office
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Lezah Roberts:

The following, in duplicate, is responsive to the office action dated 08/27/2007.

1) The Examiner argues that the term "suitable" in claim 78 is a relative term which renders the claims 78-85 and 87-92 indefinite. The Examiner indicates that the term "medicinal agent" is a definite term that does not require further description by the term "suitable." If in fact, the term "medicinal agent" is a definite term, then it is not clear why the adjective "suitable" which would enhance Applicant's requirement of the medicinal agent by enhancing the specificity of the definite term "medicinal agent" with the term "suitable" would not be useful to those skilled in the art who would follow the teachings in said application. However, since the Applicant and the Examiner are in accord that the term "medicinal agent" is a definite term in and of itself and needs no further description by the term "suitable," Applicant has amended claim 78 which removes this characteristic of the use of a "medicinal agent" and should render all claims

78-85 and 87-92 acceptable in describing with specificity that distinct claim of the subject matter which Applicant regards as his invention.

2) Applicant concedes that the term "said water-soluble acid" in claim 82 does not utilize the same term in the independent claims which relate to the term "aqueous soluble acid." In order to maintain consistency, the Examiner's suggestion is gratefully accepted in that claim 82 has been amended substituting the term "said water-soluble acid" to "said aqueous-soluble acid." It is, however, noteworthy to mention that the chemical literature and the dictionary definitions for the term "aqueous" is totally synonymous with the term "water" in that the unabridged dictionaries examined will cite the definition of "aqueous" as: of water; containing water; made with water.

3) The Examiner submits that claim 86 recites the limitation "said medicament" and that claim 86 is dependent upon claim 78 which uses the term "suitable medicinal agent." The Examiner's argument is well-taken and claim 86 has been accordingly amended.

4.

1) The Examiner submits that the instant application in claims 78-90 are rejected as being unpatentable over Cole *et al.* (US 5,089,606). With all due respect to the Examiner, merely citing that Cole *et al.* utilized some common chemicals that are common to the instant application of Scherr, fails to take into consideration certain inherent major differences between the methodology of Cole *et al.* and the instant application of Scherr.

Thus, Cole *et al.* utilized a double barrel syringe. Into each of the two barrels of the syringe are inserted two separate compounds, which then react when released through

a common exit applicator. Therefore this device is used directly *in situ* for the treatment of various pathological states.

Cole *et al.* describe their methodology with the utilization of a water-insoluble di- or trivalent metal salt. This water-insoluble di-or trivalent metal salt component is a major component in practically all of the claims of Cole *et al.* In our application we cannot possibly start with a water-insoluble di- or trivalent metal salt, since our purpose is to insolubilize the aqueous (water) soluble pectin. The use of di- or trivalent salts as practiced by Cole *et al.*, all of which are aqueous insoluble, is an attribute which would make totally unfeasible the final product in the instant application had we started with any one of those insoluble di-or trivalent metal salts. We therefore have to add a cation metal ion salt that would be capable of complexing with the aqueous soluble pectin to then form an aqueous-insoluble pectin hydrogel. These two methods are completely diverse in their chemical synthesis, their starting components, and in the methodology by which they can be used for treating a pathological state.

In the case of my pendent application we dry the composite mixture so prepared and place onto a cloth which will act as a suitable secondary dressing for the pectin product so prepared, whereas in the Cole *et al.* patent, the mixture so made in this double-barrel device has to be used 'as is' and then a secondary dressing has to be placed over it, to hold the suitable components in place.

The insoluble metal salts utilized by Cole *et al.* must be made soluble by reacting with water-soluble acids (See column 6, lines 29-35). Examples of suitable acids include alginic acid amongst others. Alginic acid is an aqueous-insoluble acid and could not possibly act as a water soluble acid neither in the patent of Cole *et al.* and certainly not in

our application, in order to convert the insoluble metal ion salts to soluble metal ion salts (emphasis added).

The Examiner admits that the reference of Cole *et al.* differs from the instant claims of Scherr in that Cole *et al.* does not disclose the preferred polysaccharide to be pectic acid; the addition of ammonium hydroxide, surfactants, plasticizers, and sodium tetraborate as well as the fact that the composition is poured onto a fibrous cloth. These differences cited by the Examiner between Cole *et al.* and the instant application of Scherr are patently significant in that Cole *et al.* by deleting them can in no way achieve the results of the dressing of Scherr and the composition by which it has been achieved.

With all due respect to the Examiner, searching the files of the U.S. Patent Office to find a series of patents where one may cite the utilization of a component or two utilized in the instant application of Scherr and another may utilize a different component used in the application of Scherr, etc. until all of the components used in the application of Scherr have been shown to be cited in a multiplicity of patents over an extended period of time, presents a distorted plethora of antecedents to the instant application which does not justify their citation as prior art, even in the hands of someone skilled in the art. Therefore, presumably anyone skilled in the art would have taken all of these individual components which exist in the composition cited by Scherr and put them all together by extracting one or two each from all of the patent applications cited in the response of the Examiner, but this has not occurred to date except in the instant application of Scherr.

The fact that the instant application of Scherr and the composition of Scherr would have been obvious to one skilled in the art begs the question in that we have not found anyone of the thousands of people who work in this area and are considered skilled

in the art to have compiled a composition as set forth in the composition of Scherr to utilize a novel bandage which when appropriately dried and placed into a sterile package provides a healthcare individual with an instant bandage to be applied to a wound or lesion as opposed to a patient making himself or herself available in an emergency room and having to wait while two separate compositions are synthesized by an appropriate chemist and they are placed in two barrels of a double barrel syringe and they are squirted onto an open wound and a gauze or other bandage put on top to hold them in place as is practiced by Cole *et al.* As a distinguished jurist once said:

> "Knowledge after the event is always easy, and problems once solved present no difficulties, - indeed, may be represented as never having had any...."

It is quite clear that a study of the research efforts of Thomas A. Edison in attempting to invent an incandescent bulb would have been obvious to anyone since it has been known for years prior to Edison that sending an electric current through a wire would cause the wire to glow. It also has been known for many, many years that burning requires oxygen, and therefore, it need not have taken over a thousand experiments for Edison with different filaments, and different chemicals, and different fibers for Edison to come to the conclusion that his fibers were burning up because he left air in his incandescent bulb and the fibers burned up the minute he let electric current through it. All he had to do was to remove the air causing a vacuum to be present after the bulb was sealed and the glowing filament would stay glowing for very, very long periods of time. Well, how then did Edison manage to get a patent on such an obvious factor if all other people would be cited against him who knew that burning requires oxygen and that sending along electric current through a wire would cause it to glow?

The fact that Cole *et al.* or any other prior inventor mentions the word "pectic acid" (Claim 2) in passing as a possible substitute for the principal product described in Cole *et al.*'s patent, without demonstrating that such an application would be feasible under the conditions set forth by Cole *et al.* doesn't in and of itself lend any more credence to the fact that Cole *et al.* mentions pectic acid and Scherr showed how pectin could be utilized and have certain unique chemical properties in order to prepare suitable medical dressings any more than Scherr should be prohibited from claiming that water is used in an application, because so many other patents happen to use water as a component in preparing unique compositions.

The mere mention in passing by Cole *et al.* and their claims 2, 10, and 14 that a polysaccharide consisting of "pectic acid" can be utilized in preparing the "water-insoluble polysaccharide hydrogel foam of claim 1" ... "wherein said salts are selected from the group consisting of calcium carbonate, calcium phosphate dibasic, barium carbonate and zinc carbonate," as is cited by Cole *et al.* in their claim 3.

The Applicant takes strong exception to the inference that the mere citation of "pectic acid" dignifies the patent of Cole *et al.* to be cited as prior art precluding the instant application of Scherr. To the contrary, it must be clearly understood that there are a plethora of pectins having varying physical and chemical properties failing which the referenced citations of Cole *et al.* would not act in the manner as claimed by Cole *et al.* Applicant respectfully cites the following volume

> *Hydrocolloid Applications - Gum technology in the food and other industries*
> by A. Nussinovitch
>
> Published by Blackie Academic & Professional (An Imprint of Chapman & Hall) in 1997

Chapter five from this volume is cited herein as Exhibit A in its entirety for the convenience of the Examiner. Note that on page 84 of said Exhibit appended hereto that the American Chemical Society has adopted specific nomenclature for different types of pectic substances as is illustrated for the definitions of pectic substances, protopectin, pectinic acids, pectin (or pectins) and pectic acid (See Exhibit A - page 85).

> "**Pectin** (or pectins) are those water-soluble pectinic acids of varying methyl ester content and degree of neutralization that are capable of forming gels with sugar and acid under suitable conditions.
>
> **Pectic acid** is a term applied to pectic substances composed mostly of colloidal polygalacturonic acids and essentially free of methyl ester groups."

Cole *et al.* (US 5,089,606) cites the use only peptic acid. All of the claims of the instant application of Scherr deal with pectin or pectins. The American Chemical Society has clearly differentiated the chemical structure as well as any combining attributes of these two chemicals in that they are uniquely different from each other. There is no more a relationship chemically, physically, and biologically between pectin and pectic acids as there would be alleged between H_2O (water) and H_2O_2 (hydrogen peroxide). In addition, the performance of pectins of the instant application is unique in their antimicrobial activity and their chemical activity as a result of the unique composition in which they constitute a component of the total matrix. Therefore, with all due respect to the Examiner, Cole *et al.* (US 5,089,606) in view of Nelson (US 4,065,614); Cole *et al.* (US 5,089,606) in view of Park *et al.* (US2001/0038831); Cole *et al.* in view of Pellico (US 4,291,025); and Cole *et al.* in view of Shah *et al.* (US 5,527,271) are not relevant as a presumption of prior art to the instant application.

These substances will vary greatly in being able to react with a di- or tri-valent

metal salt depending upon their structure and other conditions within the milieu in which they are treated. These factors are discussed in some detail in the volume entitled:

Chemistry and Function of Pectins, edited by Marshall L. Fishman and Joseph J. Jen - a part of the American Chemical Society's Symposium Series 310, wherein on page 3 (Exhibit B):

> "In all natural pectins, some of the carboxyl groups are in the methyl ester form. Depending on the isolation conditions, the remaining free carboxylic acid groups may be partly or fully neutralized, i.e., partly or fully present as sodium, potassium or D-galacturonic acid units to total D-galacturonic acid units is called the degree of esterification (DE and strongly influences the solubility, gel forming ability, conditions required for gelation, gelling temperature, and gel properties of the preparation."

The paragraph on page 7 of the volume edited by Fishman and Jen, is appended hereto as Exhibit C. The last paragraph on page 7 stipulates the important variables before one can influence the gelation of pectins, specifically with calcium and other ions.

Page 249 appended hereto as Exhibit D indicates the large number of variable pectins and the fruits, nuts, and vegetables from which they may be isolated. Page 5 of the volume edited by Fishman and Jen (Exhibit E) cites the variable physical properties of pectins which have to be observed prior to the ability of the pectins to react with di- or trivalent cation salts.

Page 9 of the volume edited by Fishman and Jen (Exhibit F) outlines the chemical properties of pectins that must be observed prior to their ability to react with di- or trivalent cation salts.

We therefore, can categorically submit that the mere mention of the term "pectic acid" by Cole *et al.* fails completely to indicate the chemical components of pectic acid

that will provide the water-soluble salts of pectic acid for the composition as set forth in the Cole *et al.* patent.

In referring to the Cole *et al.* patent, the Examiner claims that:

> "... The reference differs from the instant claims insofar as it does not disclose the preferred polysaccharide to be pectic acid; ...."

With all due respect to the Examiner, the instant application of the Applicant does not disclose the preferred polysaccharide to be pectic acid (whatever that connotation may mean). The Applicant has clearly shown the plethora of various derivatives of pectin that exist and has been specific in the instant application to indicate the specific derivatives of pectins that he has tested and feels can be utilized to be incorporated in a medical dressing of a composition as set forth in the instant application that would serve to be efficacious in treating the wound and to have certain beneficial antimicrobial and other physiological activities with a minimal of any cytotoxicity that would impair the use of the compositions of the instant application to be used in the treatment of wounds. The mere use of a term "pectic acid" discloses nothing more of a basic molecule which can exist in a considerable number of variations of chemical structure that would have varying physical and chemical properties of its own as well as varying properties when combined in the composition of the instant application.

Consequently, Cole *et al.* provides no precedent, neither when considered by itself, nor when considered in the light of Nelson (US 4,065,614), Park *et al.* (US 2001/0038831), Pellico (US 4,291,025) and Shah *et al.* (US 5,527,271) that would be sufficiently similar to the totality of the compositions set forth in the instant application of Applicant to act as a significant prior art reading on Scherr's application.

Further, to what degree those skilled in the art can roam abroad in the literature to find the specific kinds of pectic acid that could be utilized as is utilized by Scherr in the instant application can hardly be construed as prior art by Cole *et al.* even when considering the patents of Nelson (US 4,065,614), Park *et al.* (US 2001/0038831), Pellico (US 4,291,025 and Shah *et al.* (US 5,527,271) unless the specifics of the composition of Scherr resulting in gelation of the pectins used by Scherr are also utilized in the composition of Scherr for the purpose for which the instant application is designed, which is the treatment of wounds, lesions, and related pathological states.

It is particularly noteworthy that the citation of the patent of Cole *et al.* as the primary citation issued in 1992 which issue is more than 15 years later than the issued patent of Nelson and also a number of years later than the issued patent of Pellico. Therefore, if Cole *et al.* had required or had any interest in acquiring citations of prior art, for the unique composition of the instant application herein, at no time did he take advantage of the patents of Nelson or Pellico to provide the information of those chemical and physical properties of pectin which would make it possible to claim their reduction to insolubility with certain divalent or trivalent ions. It would appear that the Examiner is pleading the case of Cole *et al.* when Cole *et al.* themselves had no interest in achieving such specificity. In fact, out of the 38 examples, of compositions cited in the patent by Cole *et al.* there is not one that is concerned with those characteristics, chemical, and physical of pectin which would make feasible rendering pectin insoluble with di- and trivalent ions. We certainly know that Cole *et al.* must have been aware of the patent of Pellico since it was cited against him prior to the issue of U.S. patent number 5,089,606; and certainly as one skilled in the art as was Cole *et al.* there is an

enormous literature on the physical and chemical properties of pectins for Cole *et al.* to be cognizant of what was required to achieve insoluble pectins utilizing divalent or trivalent ions as is set forth in the patent of Cole *et al.*

The Examiner submits that Shah *et al.* disclose wound dressings comprising a hydrogel polymer impregnated into a substrate. The instant application of Scherr does not utilize wound dressings of a composition which composition are impregnated into a substrate. The composition of the instant application is layered onto a backing with no intent of impregnating the active wound dressing into the backing. The purpose of Scherr's composition is to provide a dressing which already has a backing as part of the active composition to be placed over the wound. This is particularly saving in costs and in work of having to provide a secondary backing contrary to the hydrogel polymer of Shah *et al.* where the active component is impregnated into a cloth which then requires an additional backing.

The Examiner submits that:

> "It would have been obvious to one of ordinary skill in the art to have used pectin in place of alginate motivated by the desire to use a compound with similar properties that could perform the same function as disclosed by the secondary reference."

The Examiner's characterization that pectins and alginates have "similar properties" that could perform "the same function as disclosed by the instant application" is totally contrary to the references and exhibits cited herein by the Applicant in that the basic molecular structures of alginates and pectins have widely differing physical and chemical properties and especially when modified by other ions and contained in a unique matrix as is submitted herein with the instant application, alginates would not

perform nor have the same physical, chemical as well as biological properties as the pectins for the treatment of wounds and related pathological states.

The same misunderstanding of the Examiner is true in comparing agars with pectins in which case the composition and physical and chemical properties of agars as compared to pectins differ even more radically from each other than alginates and pectin molecules (compare Exhibits A to N). These differences are accessible in the current literature and have been part of the current literature of the scientific and medical professions for many years.

The Applicant has had over 50 years of experience working with alginates and over 40 years of experience working with pectins. I must apologize in respectfully requesting that the Examiner provide two tables outlining the physical and chemical properties of alginate and pectin that can be characterized as similar and can both perform the same function as disclosed in the instant application of Scherr. The Examiner indicates that claims 78-92 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-29 of the patent of Scherr (US 6,696,077) in view of Cole *et al.* (US 5,089,606). Further, the Examiner claims that the conflicting claims are not identical, but they are not patentably distinct from each other because they disclose similar methods of making an insoluble foam. Applicant has already argued and provided sufficient evidence herein that Cole *et al.* do not disclose a process for making an insoluble pectin sponge, because an insoluble pectin sponge would require the characterization of the pectin utilized to be amenable to precipitation with certain ions failing which an insoluble pectin sponge would not result. There is no need for Applicant to restate the arguments provided previously on this issue.

Even if we were to grant the argument of the Examiner that US patent 6,696,077 and the instant application of Scherr disclose similar methods of making an insoluble foam despite the fact that the instant application herein discloses an insoluble pectin foam and US patent 6,696,077 discloses an insoluble alginate foam, albeit as we shall see shortly, not in all of the claims 1-29 of US patent 6,696,077. One can argue as to the degree that two separate chemical agents can be utilized in similar methods of making an insoluble foam and retain similar properties for the purpose for which the preparation of the insoluble foam was intended for use. Every single claim in the Cole *et al.* patent cited by the Examiner utilizes di- or trivalent metal salts to precipitate a polysaccharide. Every single claim in US patent 6,696,077 cited by the Examiner utilizes the silver ion, a monovalent metal to precipitate the alginate. The reactivity and/or the avidity of a monovalent metal such as silver in precipitating alginate has no counterpart in the different solubilities achieved when di- or trivalent metal salts are utilized to precipitate alginate. What is even more important is that the purpose of preparing a medical dressing is to utilize an active component which is released from the insoluble foam and has antimicrobial activity as is well attributed to the olygodynamic activity of silver ions. The release of a monovalent silver ion from either pectin or alginate differs in the rate of disassociation to produce the silver ion that can react with microorganisms in a wound and thereby destroy them.

The argument of the Examiner that the disclosed "similar methods in making an insoluble foam" does not consider the wholly distinct mechanisms of action – chemically and biochemically - that the methods utilized by Cole *et al.*, Scherr in his patent 6,696,077, and Scherr in his instant application. In fact, in order to provide a uniform release of silver ions from the insoluble foam of silver alginate disclosed in US patent 6,696,077 beginning

with claim 21 and inclusive to finally claim 30, Scherr has added an insoluble calcium alginate product, which in and of itself has no significant biological activity except in acting as a molecular barrier to the controlled release of the silver ions from the silver alginate product which is the basis of Scherr's patent 6,696,077. By providing within the matrix of the silver alginate, an insoluble calcium alginate moiety, it has been shown experimentally that the silver ions released from the silver alginate dressing when applied to a wound will release the silver ions in a uniform manner over a period of days which makes unnecessary the changing of the dressing every day because the inhibitory effect of the amount of silver ions released can be controlled so as they are not released in too great an amount initially and are depleted as time goes on. This is clearly shown in Exhibit G. We have added, therefore, claims predicated on the combina- tion of a metallic pectate that is desirable to also include in the matrix a calcium pectate molecule to achieve a similar attribute as shown in Exhibit G. Attached hereto, and therefore, claims 21-30 in the Scherr US patent 6,696,077 has no counterpart in the instant application of Scherr nor in any of the citations cited by the Examiner whether they be Cole *et al.* or any of the others. This is an extremely significant attribute of Scherr's US patent 6,696,077 which has been shown if otherwise neglected, could result in too quick a release of silver ions into a wound which are absorbed by a patient and resulting in a pathological state of argyria. (See Exhibit H appended hereto). Solubilities in aqueous solution of alginate and of pectins can be achieved by methods which differ radically from each other. The avidity of various ions in precipitating alginates and/or pectins vary radically from each other. The avidity will dictate the ease with which such ions are released from the alginate or pectin moieties, especially in the light of the enormous number of different alginates and pectates that exist,

and have to be specifically chosen for the purpose for which these medical dressings are utilized.

Applicant persists in pointing out that the position of the Examiner in claiming prior art, because one patent discloses the utilization of an alginate and another discloses the utilization of a pectin and claim that they therefore have similar methods of making an insoluble foam is not consistent with fact and certainly differ radically in the methods by which the insoluble foams disassociate to release the ionic precipitates of the polyaccharides wherein they differ radically in the charge they have in that no monovalent silver is utilized by any of the references cited by the Examiner in preparing a matrix disclosed by the instant application of Scherr.

Applicant calls to the attention of the Examiner, the publication in the *Journal of Wound Care* which is appended here as Exhibit J. This independent study examined ten dressings, all of which contained silver for the purpose of inhibiting microorganisms in a wound. A number of them not only contained silver, but also contained alginate, such as the Arglaes dressing shown on page 1 of Exhibit J, and the Aquacel dressing shown on page 1 contained carboxy methyl cellulose as well as silver, and the Calgitrol dressing contains a silver alginate which is the one that is before us.

In reviewing this paper it is particularly pertinent to the arguments of the Applicant that each and every one of these dressings behaved very differently in matched tests against various organisms and under various conditions of the experiment. The amount of silver contained in the dressings did not play nearly as important a part in their performance as the composition of the matrix in which the silver was contained, an

attribute which Applicant argues is the unique feature of the dressing as described in the instant application.

In at least one of the dressings, the tests showed that not only did the dressing not inhibit the microorganism, but some of the microorganisms were able to grow in and on the silver dressing, a characteristic which would literally exclude such a dressing from any application in a wound care situation.

Thus, merely containing silver is not in and of itself a proper criterion to exclude an application as being precluded by prior art. This argument is further strengthened by an examination of the plethora of different compositions and different physical and chemical properties of alginates as can be gleaned from the literature. Thus, in the book by R. H. McDowell entitled *Properties of Alginates* published in 1961, we have included in Exhibit K appended hereto, the following pages from McDowell's book – pages 3, 5, 6, 9, 10, 11, 12, and 13.

The Examiner is referred to page 96, 97, and 98 of Exhibit A which indicates the wide variety of pectins that can be made in their different chemical and physical properties.

The Kelco Company which manufactures the largest variety of alginates has documented a wide array of alginates that can be prepared and their physical and chemical properties that warrant different applications and these pages from the Kelco documents are appended here as Exhibit L.

It is quite clear that the attempts of the Examiner to relate similar functions between alginates and pectins requires their citation of specific pectins and specific alginates as well as specific hydrocolloids and we have not had in the literature any such

specificity that would preclude the revelations set forth in the instant application as constituting prior art that would preclude the instant application of Scherr.

It is particularly noteworthy to take cognizance that merely the appearance of "similarity of presumed functions" (even if we could agree on the degree to which two different compounds behave in a manner similar to each other that would preclude the claim of novelty) (Emphasis added), the particular composition of matter in which even acceptable similar compounds are compared can behave radically different as a result of the manner in which they have been compounded or prepared. The United States Food and Drug Administration understands this problem very well and mandates that specific testing be done to examine such attributes as antimicrobial activity, cytotoxicity, producing hypersensitivity, and other attributes that have to be put to the test utilizing the claimed composition. This is bo rne out very well in examining the reports of similar dressings containing silver and their varying antimicrobial activity as illustrated in Exhibit J. Another excellent example in support of Applicant's contention that the mere citation of claimed similarity between two different compounds is not sufficient in and of itself when such compounds when compounded differently will behave differently under various test conditions. An excellent example is the marked differences in cytotoxicity reported for a number of silver dressings having many common compositions (see Exhibit M appended hereto).

The performance therefore, of two dressings which have some similar compounds as part of the dressing can have attributes on performance that would readily negate one as acting as a predicate device precluding the novelty of improved performance and safety of a subsequent product, contrary to the position taken by the Examiner.

> Section 35 § 103, PT II, CH. 10, PATENTABILITY OF INVENTIONS is intended to articulate a standard of invention somewhat less strict than that implied by reference to flash of creative genius but also to remind that patentability should be judged against state of art at time invention was made without benefit of hindsight predicated on subsequent art. (Frantz Mfg. Co. v. Phenix Mfg. Co., C.A. Wis.1972, 457 F.2d 314). The intent of this section also is not that either Examiner, Board of Appeals, or Court of Customs and Patent Appeals [now Court of Appeals for the Federal Circuit] should substitute their own speculations for factual knowledge of those skilled in the art. (Application of Katzschmann, 1965, 347 F.2d 620, 52 CCPA 1497).

The only person skilled in the art that meets the criteria of the instant application of Scherr is Scherr. None of the references cited by the Examiner, in part or considered together, constitute the composition of Scherr. If the Examiner can specifically cite those pectins which behave similarly to those cited in the application of Scherr and those alginates which can be considered in performance to the pectin ingredients in the composition of Scherr, then that would have to be examined in total to determine that the performance of those is similar to those performed by the composition of the instant application.

The Examiner cites the pertinent quotation from 35 U.S.C. § 103(a) and it is particularly pertinent to repeat the quotation cited by the Examiner:

> "(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made."

There are two attributes of this section 35 U.S.C. § 103(a) which require emphasis and they are:

"subject matter as a whole"

And

"...Patentability shall not be negatived by the manner in which the invention was made."

With regard to Cole *et al.* (US 5,089,606) the Examiner admits that the references differ from the instant claims insofar as they do not disclose the preferred polysaccharide to be pectic acid; the addition of ammonium hydroxide, surfactants, plasticizers and sodium tetraborate; and further that the composition is poured onto a fibrous cloth.

With regard to the reference to the patent by Nelson (US 4,065,614), the Examiner admits that this reference differs from the instant claims insofar as it does not disclose the pectins are used in a sponge or foam for a wound dressing or a process for making the wound dressing.

With regard to the citation of the patent of Pellico (US 4,291,025) as indicated by the Examiner, Pellico's patent differs from the instant claims insofar as it does not disclose a procedure for making a insoluble pectin sponge or foam comprising adding ammonium hydroxide, an effervescent compound, and acid followed by pouring the solution onto a fibrous cloth and drying the composition.

The Examiner also cites the patent of Shah *et al.* (US 5,527,271) and admits that the reference of Shah *et al.*'s patent differs from the instant claims insofar as it does not disclose the hydrogel is a insoluble pectin sponge or foam using a metal ion salt, acid, an effervescent agent, ammonium hydroxide and sodium tetraborate.

None of these references cited by the Examiner as constituting prior art fulfill the criterion of 35 U.S.C. § 103(a) in that the subject matter as a whole is clearly not obvious at the time that these citations of ostensibly prior art were made and have not been included as a whole in any of these citations of the Examiner, despite the fact that the citations represent the work of individuals who are skilled in the art. In addition, the

manner in which the instant application is made, the invention cited herein by Scherr has no counterpart to any one of the prior art cited by the Examiner.

The Examiner submits that "claims 91-92 are rejected as being unpatentable over Cole *et al.* (US 5,089,606) in view of Nelson (US 4,065,614), Park *et al.* (US 2001/0038831, Pellico (US 4,291,025) and Shah *et al.* (US 4,427,271) as applied to claims 78-90 above, and further in view of Bannert (US 5,147,648)".

The Examiner admits that the primary and secondary references differ from the instant claims of Scherr insofar as they do not disclose that mixtures of polysaccharides are used to make the gel compositions. Bannert discloses the advantage of using a mixture of polysaccharides. However, Bannert differs from the instant claims of Scherr as it does not disclose the compositions are made in to a foam wound dressing by adding acid, an effervescent compound, ammonium hydroxide and tetraborate to the compositions and further, which are poured on to a fibrous cloth. These omissions again are significant omissions and fail to meet the criteria of 35 U.S.C. § 103 in that the subject matter of Bannert as a whole differs radically from the instant claims of Scherr. Since the instant claims of Scherr have the capability, as set forth in the specification of Scherr, that:

> "The use of desirable particulate matter such as micro-particles that can act as time-release particles, aqueous insoluble medicaments, or even the use of intact cells such as yeast cells, blood cells, or human or animal tissue cells, that might be desirable to apply to an open wound may be introduced into the pectin foam composition described herein."

Thus the failure of Bannert to prepare a composition of matter in the form of a foam obviates completely the ability of Bannert and all of the other references cited by the Examiner who have failed to utilize the composition that would make it possible for

the inclusion of particulate matter or the inclusion of aqueous insoluble medicaments or even the use of intact cells as indicated to be feasible in the instant application of Scherr.

We respectfully submit that the amended claims are as follows:

Amended claim 78:

78. A process for making an aqueous-insoluble cation cross-linked pectin sponge or foam product to be utilized in the preparation of wound dressings or surgical products comprising the steps of:

(I) making an aqueous solution of an aqueous-soluble pectin composition in which the pectin has a degree of esterification of less than 50% and a degree of amidation of less than 50%;

(II) while allowing the total composition of (I) to be mixed, adding a cation metal ion salt capable of complexing the aqueous-soluble pectin to form an aqueous-insoluble pectin hydrogel;

(III) adding to the mixture of (II), a plasticizer, a surface active agent, sodium tetraborate, ammonium hydroxide, and a ~~suitable~~ medicinal agent;

(IV) while continuing to mix the entire composition (III), adding an aqueous-soluble effervescent compound capable of effervescence upon reaction with an aqueous-soluble acid;

(V) adding to the composition with continued mixing (IV) an aqueous-soluble acid;

(VI) pouring said composite mixture of (V) onto a fibrous cloth contained in or on a tray, which fibrous cloth will become affixed to the foamed pectin

composition as a backing after the aqueous component of said composite mixture has evaporated.

Amended Claim 82:

82. The process of claim 78 wherein said ~~water-soluble acid~~ aqueous-soluble acid is selected from the group consisting of acetic, lactic, malic, gluconic, hydrochloric, and ascorbic acids.

Amended Claim 86:

86. The process of claim 78 wherein said ~~medicament~~ medicinal agent is selected from the group consisting of collagen, maltodextrin, antibiotics, antibacterial agents, anti-inflammatory agents, ascorbic acid, amino acids, and mixtures thereof.

The following claims are withdrawn:

Claim 96 and,

Claim 97.

The following new claims are respectfully submitted:

108. A silver pectate wound dressing affixed to a fibrous cloth backing.

109. A silver pectate-calcium pectate wound dressing affixed to a fibrous cloth backing.

110. A silver pectate wound dressing.

111. A silver pectate-calcium pectate wound dressing.

112. A copper pectate wound dressing affixed to a fibrous cloth backing.

113. A copper pectate-caclium pectate wound dressing affixed to a fibrous cloth backing.

114. A copper pectate wound dressing.

115. A copper pectate-calcium pectate wound dressing.

116. An iron (Fe++ or Fe+++) pectate wound dressing affixed to a fibrous cloth backing.

117. An iron (Fe++ or Fe+++) pectate-calcium pectate wound dressing affixed to a fibrous cloth backing.

118. An iron (Fe++ or Fe+++) pectate wound dressing.

119. An iron (Fe++ or Fe+++) pectate-calcium pectate wound dressing.

120. A silver pectate wound dressing containing a medicament affixed to a fibrous cloth backing.

121. A silver pectate-calcium pectate wound dressing containing a medicament affixed to a fibrous cloth backing.

122. A silver pectate wound dressing containing a medicament.

123. A silver pectate-calcium pectate wound dressing containing a medicament.

124. A copper pectate wound dressing containing a medicament affixed to a fibrous cloth backing.

125. A copper pectate-calcium pectate wound dressing containing a medicament affixed to a fibrous cloth backing.

126. A copper pectate wound dressing containing a medicament.

127. A copper pectate-calcium pectate wound dressing containing a medicament.

128. An iron (Fe++ or Fe+++) pectate wound dressing containing a medicament affixed to a fibrous cloth backing.

128. An iron (Fe++ or Fe+++) pectate-calcium pectate wound dressing containing a medicament affixed to a fibrous cloth backing.

129. An iron (Fe++ or Fe+++) pectate wound dressing containing a medicament.

130. An iron (Fe++ or Fe+++) pectate-calcium pectate wound dressing containing a medicament.

131. A silver pectate moiety.

Applicant submits that the application should now be considered for issue.

Respectfully submitted,

George H. Scherr, Ph.D.

GHS/jj

Hydrocolloid Applications

Gum technology in the food and other industries

by
A. Nussinovitch

Published in 1997
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5 Pectins

5.1 Introduction

The natural polymers (structural materials) found in all land plants are termed pectins (Braconnot, 1825). Like starch and cellulose, pectin is a structural carbohydrate (Christensen, 1986). It was discovered in the 18th century (Vauquelin, 1790), and Braconnot was the first to characterize it as the active fruit component responsible for gel formation. He also suggested the word 'pectin', which originates from a Greek word meaning 'to congeal or solidify'.

Commercially, pectin is extracted from citrus peel or apple pomace. The commercial isolation of pectins from suitable plant material began early in the 20th century and has been developing ever since. Pectic substances are integral structural components of the cell and play an important role as cementing material in the middle lamellae (Fig. 5.1) of primary cell walls (Christensen, 1986). The many reviews and comprehensive texts on pectin are valuable sources for the reader (Kertesz, 1951; Doseburg, 1965; Pilnik and Zwiker, 1970; Christensen and Towles, 1973; Pedersen, 1980; May, 1992; Sakai *et al.*, 1993). The release of pectin involves acidic extraction and isolation by precipitation, followed by drying to obtain a powder with standard properties. Ultrasound has been suggested to intensify pectin de-esterification (Panchev *et al.*, 1994). Pectins are normally dried to less than 10% water content. The product is kept in a vapor-tight package under cool, dry conditions. Commercial pectins usually have particle sizes of ~ 0.25 mm and a low density, $\sim 0.7\ g\ cm^{-3}$. Commercial pectins include mainly polymerized galacturonic acid that has been partly esterified with methanol (Rolin and De Vries, 1990). The percentage of the partially esterified portion of polymerized galacturonic acid strongly influences the functional properties of the pectin, and pectins with both low and high ester contents are sold. At low pHs pectins with high ester contents with the addition of enough sugar create fruit-system gels (Rolin and De Vries, 1990).

Pectin is used as a gelling agent in traditionally manufactured fruit-based products, especially jams and jellies. The heat stability of pectin under acidic conditions makes it an ideal candidate for the conditions occurring when texturization or stabilization are required in acidic food systems. Home-made jam-making is based on the fruit pulp's ability to form gels when boiled with sugar: the natural pectin content in the pulp is responsible for the gelation. Commercial jam processing adds already produced pulp,

Exhibit A

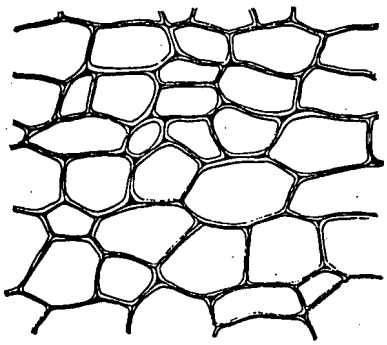


Figure 5.1 The middle lamella seen as a solid black mass between the cells of an unripe apple (× 1350), adapted from *The Pectic Substances* by Z.I. Kertesz (1951), Interscience Publishers, New York.

yielding more uniform preparations. High-ester pectins can form gels at low pH when sufficient amounts of sugar are added (thereby reducing water activity in the system). Low-ester pectins create gels in the presence of calcium ions via a different mechanism. The increasing amounts of pectins produced are now utilized outside their traditional industry as part of the confectionery industry, as stabilizers in the milk industry, and for pharmaceutical purposes.

5.2 Nomenclature

Pectin and pectic substances are heteropolysaccharides consisting mainly of galacturonic acid and galacturonic acid methyl ester residues (Christensen, 1986). To obtain uniform definitions in this area, the American Chemical Society adopted a revised nomenclature for pectic substances (Baker *et al.*, 1944) as follows.

Pectic substances are those complex colloidal carbohydrate derivatives that occur in or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units, which are thought to exist in a chain-like combination. The carboxyl groups of polygalacturonic acid may be partly esterified by methyl groups and partly or completely neutralized by one or more bases (Christensen, 1986).

Protopectin is the water-insoluble parent pectin substance that occurs in plants and which, with restricted hydrolysis, yields pectin or pectinic acid.

Pectinic acids are the colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups. Pectinic acids, under

suitable conditions, are capable of forming gels in water with sugars and acid, or if suitably low in methoxyl content, with certain ions. The salts of pectinic acids are either normal or acid pectinates.

Pectin (or pectins) are those water-soluble pectinic acids of varying methyl ester content and degree of neutralization that are capable of forming gels with sugar and acid under suitable conditions.

Pectic acid is a term applied to pectic substances composed mostly of colloidal polygalacturonic acids and essentially free of methyl ester groups.

Protopectinase is the enzyme that converts protopectin into a soluble product. It is also called pectosinase or propectinase (Christensen, 1986).

Pectinesterase (PE) or pectinmethylesterase is the enzyme that catalyses the hydrolysis of the ester bonds of pectic substances to yield methanol and pectic acid. The name *pectase* does not indicate the nature of the enzyme action and has, therefore, been replaced by these more specific names.

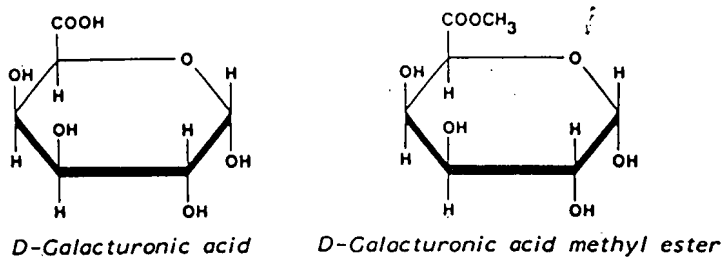
Polygalacturonase (PG) or pectin polygalacturonase is the enzyme that catalyses the hydrolysis of glycosidic bonds between de-esterified galacturonide residues in pectic substances.

Pectinase is frequently used to designate the glycosidase as well as pectic-enzyme mixtures (Baker *et al.*, 1944; Christensen, 1986).

A modern definition of pectin takes into consideration the low methyl ester content and the amidated pectinic acids as follows: pectin is a complex, high-molecular-weight polysaccharide consisting mainly of the partial methyl esters of polygalacturonic acid and their sodium, potassium and ammonium salts. In some types (amidated pectins), galacturonamide units further occur in the polysaccharide chain. The product is obtained by aqueous extraction of appropriate edible plant material, usually citrus fruit and applies (Christensen, 1986).

5.3 Structure

Commercial pectins are composed mainly of polymerized, partly methanol-esterified (1–4)-linked α-D-galacturonic acid. The pectin molecule can contain 200–1000 linked galacturonic acid units. In some pectins, the methyl ester groups are partially replaced by amide groups, to a maximum of 80% (Fig. 5.2). During extraction, only part of the pectin molecules can be extracted by non-degradative means, whereas dilute acids are generally used



D-Galacturonic acid *D-Galacturonic acid methyl ester*

Figure 5.2 Principal units in the pectin molecule.

(Rolin and De Vries, 1990). Therefore, the structure of the resultant pectin differs greatly. About 5–10% of the galacturonic acids are neutral sugars such as galactose, glucose, rhamnose, arabinose and xylose. They can be bound to the galacturonate main-chain, be inserted into the main-chain (rhamnose) or be a part of contaminating polysaccharides (glucans and xyloglucans). Pectins from apple, citrus, cherry, strawberry, carrot, pumpkin, sugar beet, potato, onion and cabbage have the same neutral sugar composition (Amado and Neukom, 1984; Guillon *et al.*, 1986; Rolin and De Vries, 1990), in contrast to pectins from mountain pine pollen, Japanese kidney beans and duckweed, which contain large amounts of xylose or apiose (Mascaro and Kindell, 1977; Matsuura, 1984). Bacterial enzymes can be used to extract pectin from pumpkin and sugar beet (Matora *et al.*, 1995). Information on the characterization of pectic substances from selected tropical fruits such as orange, lime, banana, mango, avocado, pawpaw, cashew apple, star apple, tomato and guava, in terms of their gelation properties, can be found elsewhere (Nwanekezi *et al.*, 1994).

X-ray diffraction studies performed on dried fibers to study the structure of pectin indicated that the galacturonan backbone forms a right-handed helix, with three galacturonic acid units in C_1 conformation as the repeating sequence, corresponding to a repeat distance of 1.34 nm (Palmer and Hartzog, 1945; Walkinshaw and Arnott, 1981a). Morris *et al.* (1982) suggested that gel formation with calcium involves polygalacturonic acid sequences with a 2_1, ribbon-like symmetry. Upon drying the gel, however, the 3_1 helical symmetry is restored via polymorphic phase transition. Commercial pectins contain lower amounts of neutral sugars relative to pectin extracted under mild conditions. A large proportion of these sugars is 1,2-bound rhamnose present in the galacturonan backbone. However, rhamnose's distribution along the pectin chains has not yet been fully elucidated (Christensen, 1986). The length of polygalacturonate sequences between rhamnose interruptions has been suggested to be fairly constant, corresponding to ~25 residues (Powell *et al.*, 1982). Analysis of similar sequences with 20 to 30 degrees of polymerization (Neukom *et al.*, 1980) showed both oligomers to be almost fully made up of galacturonic acid units

with only traces of rhamnose, and galacturonan segments containing rhamnose from apple tissue. Therefore, it was concluded that cell walls can contain both a pure galacturonan-type pectin and a rhamnogalacturonan-type pectin in different regions. A molecular model of their blockwise occurrence in a few hairy regions was suggested, based on results obtained by the specific enzymatic degradation of apple pectins (De Vries *et al.*, 1982).

5.4 Sources and properties

Pectins can differ as a result of ripening and these differences can influence the efficiency of the extraction process (De Vries *et al.*, 1984; Huber, 1984; Boothby, 1983). Pectin extracted from the primary cell wall may have more branches of neutral sugars than that extracted from the middle lamella (Redgwell and Selvendran, 1986). Side-chains (neutral sugar side-chains) are distributed unevenly along the main-chain. Therefore, models describing smooth and hairy regions within pectin that has been extracted by a mild process can be deduced for pectic substances from citrus, sugar beet, cherry and carrot (Rolin and De Vries, 1990).

A description of pectic fractions from different sources can be found elsewhere (Rolin and De Vries, 1990). Substituents such as acetyl groups (in potato and sugar beet pectins) can prevent gelation. In apple and citrus, only a very low degree of acetylation is measured, and the acetyl groups may be located in the hairy regions (Vorgen *et al.*, 1986). Active pectin oligomers have also been detected in ripening tomato fruits (Melotto *et al.*, 1994). Moreover, associations of pectin with boron in the cell walls of squash and tobacco have been reported (Hu-Hi and Brown, 1994). Since pectin can come from different agricultural sources, it is not surprising that different pectins have different substituents located in different positions. Recently, pectin has been extracted from Galgal (*Citrus Pseudolimon Tan*) (Attri and Maini, 1996). The process was standardized for maximum recovery of pectin from these peels using various extractants and varying the extractant, peel ratio, extraction time, number of extractions and peel particle size (Attri and Maini, 1996). Pectin extraction from citrus peel using PG produced on whey has been reported (Donaghy and Mckay, 1994). Dried sweet whey was used as a complete medium for the production of the enzyme by the yeast *Kluveromyces fragilis*. The concentrated enzyme was then used to release pectin from the peels and apple pomace but was unable to release pectin from sugar beet pulp. Conditions for pectin extraction from orange peel were optimized with regards to enzyme concentration, water:peel ratio, temperature and duration of treatment (Donaghy and Mckay, 1994).

The most abundant substituent is the methanol ester of galacturonate residues. If apple or citrus pectins are not subjected to de-esterification, their

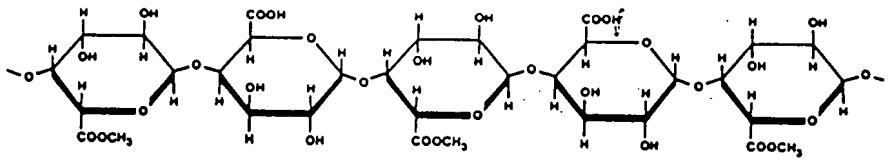


Figure 5.3 Section of a high ester content pectin molecule, with a DE ~60%.

degree of esterification (DE) is high (~70%) compared with the low DE values for pectins extracted from sunflower heads, potato, tobacco and pear (Vorgen *et al.*, 1986; Turmucin *et al.*, 1983; Pathak and Shukla, 1981). The DE is defined as the ratio of esterified galacturonic acid units to the total number of galacturonic acid units in the molecule (Fig. 5.3). These values can also be influenced by the degree of ripening of the raw material and changes in the extraction procedure. Ester group distribution depends on the source. There is evidence of random intramolecular distribution in mildly extracted apple pectins, contradicting a work which reported some regularity (De Vries *et al.*, 1983 and De Vries *et al.*, 1986, respectively). Non-random distribution has been reported in commercial pectins (Anger and Dongowsky, 1984, 1985; De Vries *et al.*, 1986). Information on de-esterification by fungal enzymes, pectin structure, conformation in solution and gels, and other properties can be found elsewhere (Kohn *et al.*, 1983, 1985; Markovic and Kohn, 1984).

The main sources of commercial pectins are citrus (lemon, lime, orange and grapefruit) peel and apple pomace. Peels are supplied for pectin production after the juice has been squeezed and the essential oils extracted. After conveying the peels to the extraction site, a water wash is used to remove as much water-soluble material as possible, other than pectin, and then extraction is begun or the peel is dried for future processing. It is not surprising to find pectin plants near plants that can supply them with the raw material directly, such as those producing apple or citrus juice, or cider. Apple pomace, which once served as the major raw material, has been replaced to a large extent by citrus peel because the latter contains 15–20% more pectin on a dry weight basis. During the Second World War, sugar beet waste (from sugar production) served as a source of pectin production. Since this pectin contains acetyl ester, other better sources are preferred. As mentioned earlier other raw materials exist (Pathak and Shukla, 1978).

5.5 Pectin manufacture

Pectin manufacturing processes are generally known (Fig. 5.4). However, variations in, or fine-tuning of, the processes, i.e. the specific conditions used, are kept confidential by the manufacturers who consider them trade secrets.

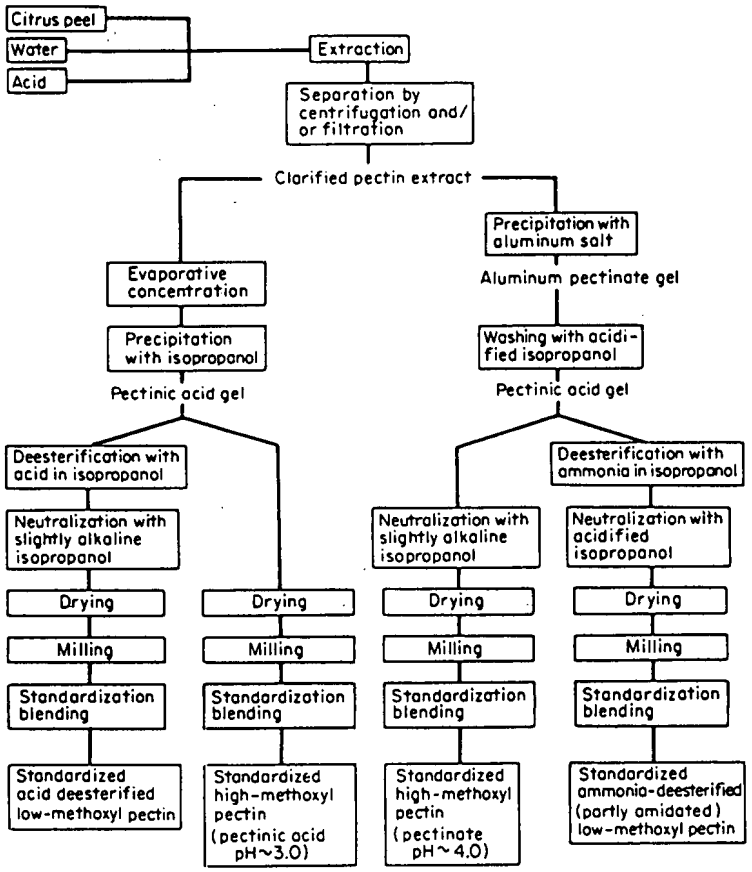


Figure 5.4 Various process routes for the manufacture of pectins. (Adapted from *Handbook of Water-Soluble Gums and Resins*, McGraw-Hill, NY, 1980, R.L. Davidson, ed.)

In general, the fresh or dried raw material (apple pomace, citrus peel and a number of other surplus materials such as sunflower bottoms and sugar beet waste) (Karpovich *et al.*, 1981) is extracted in demineralized water that has been acidified with mineral acid to give a pH of 1.5 to 3.0 (hydrochloric or nitric acid are most often used) at 70°C and for ~3 h. In the case of citrus peel, pretreatment of the peel by blanching and washing to eliminate PE activity and to remove glucosides, sugars and citric acid is common. Dried peel is stable under storage conditions, making its transport over great distances feasible. Dried citrus peel contains ~20–30% pectin. Dried apple pomace yields 10–15% pectin.

Since a certain degree of pectin de-esterification takes place during the extraction, conditions should be chosen to fit the desired product (Rolin and De Vries, 1990). Temperature, pH and time need to be carefully controlled.

Rapid-set high-ester pectins are generally extracted at temperatures close to boiling. At these high temperatures, hydrolysis of the parent pectic substances accelerates, viscosity is lowered and diffusion facilitated. This process may take less than 1 h with only minor de-esterification, whereas lower extraction temperatures and longer extraction times favor de-esterification to yield slow-set high-ester pectins or even low-ester pectins. After separating out the extract, the peels can be used as cattle feed, while the extracted liquid (viscous liquid containing 0.3–1.5% dissolved pectin) is clarified by filtration and centrifugation. At this stage, the clear pectin extract can be further de-esterified by maintaining controlled pH and temperature. The extract can be concentrated and, after preservation with sulfur dioxide, sold as 'liquid pectin'.

Pectin can be isolated by alcohol precipitation or by precipitation as an insoluble salt. With the former procedure, the pectin (unlike the water-soluble materials in the extract) precipitates out, and the alcohol is recovered by distillation. Alcohol precipitation is effected by mixing the extract with methanol, ethanol or 2-propanol. In some processes, the extract is concentrated by evaporation prior to precipitation to minimize distillation costs (Rolin and De Vries, 1990). Other alternative procedures involve pectin separation by aluminum or copper ions as an insoluble salt (Kausar and Nomura, 1982; Michel *et al.*, 1981). These metal ions can later be removed by acidified alcohol washes then a wash in alkaline alcohol to neutralize the product. The resultant alcohol-wetted pectin is pressed, dried and milled, or is de-esterified in the alcohol suspension (Rolin and De Vries, 1990).

De-esterification can be achieved with an acid or base. If ammonia is used, then some of the methyl ester groups are replaced by amide groups and the product is termed 'amidated pectin'. For pectin manufacture, as for other hydrocolloids, a blending and standardization stage is important. With the inclusion of this stage, the marketed blends exhibit very similar performance with respect to firmness of the resultant gel and the time necessary to gel high-ester pectins under predetermined constant conditions. In a similar manner, low-ester pectins are standardized in terms of their calcium reactivity.

Pectin manufacturers are located in many places worldwide. A few examples are Hercules (factories in Denmark, Germany and Florida, USA), Unipectine (France), Pektin-Fabrik (Germany), General Foods Corp. (USA) and Pectina de Mexico. Smaller pectin manufacturers are found in Switzerland, Brazil, Israel, Argentina and a few other European countries.

5.6 Commercial availability, specifications and regulatory status

Descriptions and terminology of commercial pectins exist (Doesburg, 1965). Commercial pectin is defined as the partial methyl esters of polygalacturonic

acids and their sodium, potassium, calcium and ammonium salts. The pectin is extracted from edible plant organs and no organic precipitants other than methanol, ethanol and isopropanol are used. Amidated pectins can be produced by ammonia treatment. Standardization can be achieved by dilution with sugars. Buffer salts are permitted to yield desirable setting conditions.

Commercial pectins are divided into high- and low-ester pectins in accordance with their DE values: a value over 50% is considered a high-ester pectin, values from 50% to negligible amounts define a low-ester pectin (Rolin and De Vries, 1990).

Pectate is a polymerized galacturonic acid with no or only negligible esterification. The degree of amidation (DA) is the percentage of galacturonic acid subunits that are amidated. High-ester pectins used for gel-making can be divided into rapid-set, medium-set and slow-set pectins, depending on the time necessary for solidification. The higher the DE (in high-ester pectins), the shorter the setting time. High-ester pectins are regularly standardized to 150 grade of USA-SAG, meaning that 1 part pectin can solidify 150 parts of sucrose into a jelly with the standard properties of 65° Brix (soluble solids), pH 2.2–2.4 and 23.5% SAG (indication of gel strength, see section 5.8.1).

In addition to the definitions of commercial pectins, purity is also defined by several requirements: galacturonic acid content $>65\%$, DA $<25\%$, loss on drying not more than 12%, acid-insoluble ash not more than 1%, alcohol residues of all kinds not more than 1%, nitrogen not more than 2.5% and sulfur dioxide not more than $50\,mg\,kg^{-1}$ (Food Chemicals Codex, 1981; Anon., 1978; Anon., 1981a,b). Since pectin is an important constituent of land plants, it is consumed in significant quantities. Pectin passes unchanged (no enzymatic degradation) to the large intestine, where bacteria use it as a carbon source. However, its hydrolysation in the intestinal tract produces next to no calories (Cambell and Palmer, 1978). From a toxicological point of view, there are no limitations on its use (Anon., 1981a,b). Pectins are GRAS for use in human foods. The FDA has not issued any specific limitations or guidelines for their use in any food (Anon., 1981c). The potential dietary benefits of citrus pectin and fiber have been reviewed by Baker (1994). Other health aspects of pectin are important and have been studied by many researchers. Examples include the role of pectin in cholesterol regulation (Cerda, 1994), citrus pectin and cholesterol interactions in the regulation of hepatic cholesterol homeostasis and lipoprotein metabolism in the guinea pig (Fernandez *et al.*, 1994), the use of pectin as a fat replacer (Hoefler, 1994), oral administration of modified citrus pectin as an inhibitor of spontaneous prostate cancer metastasis in rats by inhibiting carbohydrate-mediated cell–cell interactions (Pienta and Raz, 1994), the dose response of colonic carcinogenesis to pectin and guar gum (Klurfeld *et al.*, 1994), and the effects of structural parameters of pectin on its interaction with drugs *in vitro* (Fritzsch *et al.*, 1994). Recently, a pectin-supplemented

enteral diet was reported to reduce the severity of methotrexate-induced enterocolitis in rats (Mao *et al.*, 1996). Information on the preparation and physicochemical properties of polymer complexes of benzimidazolyl-2-methylcarbamate and apple pectin can be found (Khalikov *et al.*, 1995), and a new pectin-based material for selective low density lipoprotein-cholesterol removal has been reported (Lewinska *et al.*, 1994). The physiological effect of low-molecular-weight pectin is discussed by Yamaguchi *et al.* (1994). Such pectins that can retain their activities are important since high viscosity reduces their usability. This preparation exhibited high solubility and a repressive effect on lipid accumulation in the liver (Yamaguchi *et al.*, 1994). Pectin formulations have also been used for colonic drug delivery (Ashford *et al.*, 1994).

High-methoxy pectin loses about 5% of its USA-SAG grading when stored at 20°C in a dry atmosphere, whereas low-ester pectin is more stable and under favorable conditions loss is undetectable (Food Chemicals Codex, 1972; Anon., 1978; Anon., 1981c). The microbiological purity of pectins is specified in many cases by the manufacturer, since it is used mainly in acidic media and, therefore, yeast and mold counts are relevant. Typical specifications may include a total plate count at 37°C of less than 500 cells g^{-1}; a yeast and mold count at 25°C of less than 10 cells g^{-1} and *Escherichia coli*, salmonella and staphylococcus test results being negative.

5.7 Solution properties

Good pectin solubility can be achieved by following recommended dissolution procedures. In general, pectin is not soluble under conditions in which it forms a gel. The powder needs to be dispersed in warm water (not less than 60°C), at reduced mixing rates and then at full speed. Ignoring manufacturer recommendations could result in the formation of lumps that are difficult to dissolve. Good dissolution is achieved by mixing pectin with five times its own weight of sugar. Other blending media, such as a 65% sugar solution or alcohol to wet the pectin for small-scale laboratory use, are recommended. If a high-shear mixer is not used, then boiling for 1 min is necessary to guarantee full dissolution (Rolin and De Vries, 1990).

5.7.1 Viscosity

The viscosity of pectin solutions is dependent on their concentration, presence of calcium or similar non-alkali metals, pH, the chemical properties of the pectin, the DE and the average molecular weight. Dilute pectin solutions (up to approximately 0.5%) are Newtonian and only slightly affected by calcium ions. Increased pH results in increased viscosity. Salts of monovalent cations reduce pectin solution viscosity, because of reductions

at high ionic strength. The higher the average molecular weight, the higher the solution viscosity. The molecular weight of pectin can be estimated by using intrinsic viscosity methods. Pseudoplastic solutions can be achieved with concentrations higher than 1%. In contrast to dilute solutions and in the absence of calcium, such solutions increase in viscosity if the pH is reduced within the typical application range of 2.5–5.5. Pectins in the presence of calcium form thixotropic solutions, the viscosity of which increases with increasing pH within the aforementioned range. In fact, different textures can easily be achieved by combining pectin types and concentrations, ion concentrations and pH (Michel *et al.*, 1982; Christensen, 1954; Berth *et al.*, 1982). Solution properties of pectins are changed by hydrolysis of side-chains. Hydrolysis did not affect the specific viscosity of dilute (0.5%) pectin solutions; however, viscosity significantly decreased in concentrated 2.0–6.0% pectin solutions. Results suggest that pectin side-chains exist in an entangled state in concentrated solutions. In these latter solutions, the extent of viscosity reduction was dependent on pectin concentration (Hwang and Kokini, 1995). Based on viscometry measurements, the average molecular weight of commercial pectin normally falls between 50×10^3 and 150×10^3. It is important to note that by using other techniques such as light-scattering, other results ($\sim 1 \times 10^6$ or higher) have been found owing to intermolecular associations and aggregation of pectin molecules.

5.7.2 Chemistry and properties

Pectin is in fact a polyacid. The negative charge on dissolved pectin is smaller at low pH than at high pH. This charge attracts protons and, therefore, dissociations of individual acid groups are not independent (Rinaudo, 1974). Via a mechanism known as 'membraneless osmosis', pectin can concentrate solutions of proteins such as milk proteins. Since pectin and the protein cannot exist in the same solution, two phases develop, one rich in pectin and the other in protein. Pectin has a higher affinity for water and the protein phase is thus concentrated by a factor of 5–12. The addition of metal ions to pectin solutions causes an increase in viscosity, or gel formation or pectin precipitation. Reactions of polyanions (pectin) with polycations (other macromolecules) form insoluble products. Dissolved pectin exhibits good stability at pH 4. Far from this optimum, depolymerization occurs at low pH, whereas at high pH (any pH > 5) degradation occurs owing to β-elimination. High-ester pectins are more vulnerable to such degradation than their low-ester counterparts. In the juice industry, pectin-degrading enzymes are often used to obtain a clarified product.

A study of synergistic interactions in dilute polysaccharide solutions was recently performed (Goycoolea *et al.*, 1995). A simple viscometric approach was used in cases in which exclusion effects should be negligible. There were

no viscosity changes for alginate and pectin with sufficient calcium ions to induce almost complete conversion to the dimeric 'egg box' form, demonstrating that conformational rigidity is not, in and of itself, sufficient for other polysaccharides to form heterotypic junctions with mannan or glucomannan chains.

Atomic force microscopy (AFM) was used for imaging polysaccharides such as pectin, ι-carrageenan, xanthan and acetan (Kirby *et al.*, 1996). The polysaccharides were deposited from an aqueous solution onto the surface of freshly cleaved mica, air-dried and then imaged under alcohols. Improved resolution was obtained relative to the more traditional metal-coated samples or replicas (Kirby *et al.*, 1996).

5.8 Pectin gels

High-ester pectin gels can be successfully prepared following good dissolution. Jam preparation procedures can be found elsewhere. Briefly, they include heating the sugar and fruit fraction in amounts that will yield 65% soluble solids in the final batch. The pectin is added in solution form and stirring and boiling are carried out under vacuum to achieve the desired soluble-solids content. The vacuum needs to be broken before heating to pasteurization. Then citric acid is added to reduce the pH to 3.0–3.1. The mixture is cooled to filling temperatures and gelation occurs in the container itself. For high-ester pectin gelation, low pH, a high soluble-solids concentration and appropriate temperatures are needed to fulfill the desired requests. A high-ester pectin gel cannot be melted after solidification. Pregelation phenomena (stirring while gelation is in progress) result in lower-strength gels, or the absence of gelation with continued interference. For gel formation, a three-dimensional network is necessary to hold water, sugar and other solutes (Fig. 5.5). The junction zones in the high-ester pectin gel network have been described by a model suggested by Walkinshaw and Arnott (1981b). According to this model, three to ten polymer-chain segments with a helical structure form aggregates of parallel chains that are limited in size because of steric barriers, entropic factors and possibly rhamnose insertions (Christensen, 1986). Local crystallization is sustained by intermolecular hydrogen bonds and is probably reinforced by hydrogen bonding with water molecules in one set of triangular channels, and hydrophobic attractions between methyl groups forming columns in a second set of triangular channels. In molecular gel networks, at least two types of bonding are involved. One is strong and responsible for the elastic properties of the gel and the other is weaker and capable of reforming after disruption. Sugars play an active role in the formation of the pectin gel network by associating with pectin molecules via hydrogen bonding to form secondary links that reinforce the molecular network structure. The aging

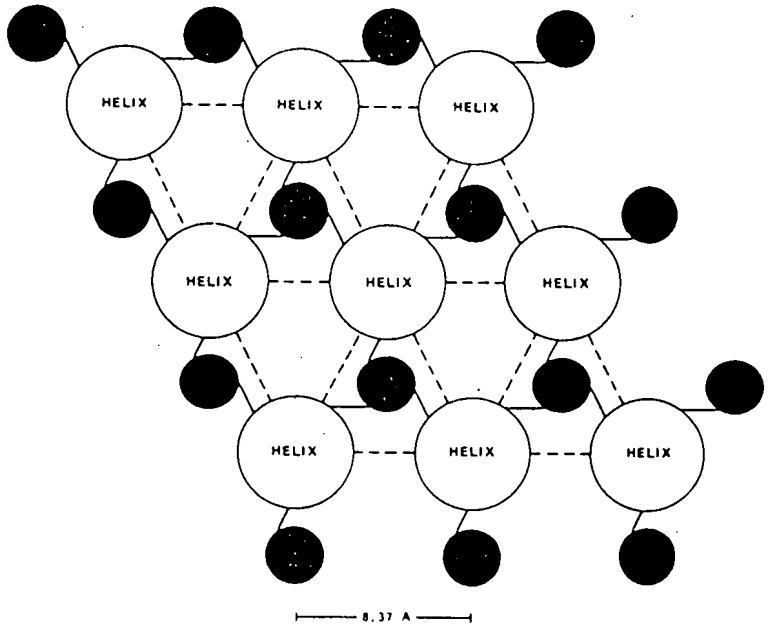


Figure 5.5 Junction zone in a high-ester pectin gel. (From Walkinshaw and Arnott, 1981b.)

process of high-methoxy pectin (HMP)–sucrose aqueous gels can be followed by low amplitude oscillation (Dasilva and Goncalves, 1994). Dynamic mechanical measurements enabled a determination of the point at which the system undergoes the sol–gel transition. The HMP–sucrose system is extremely sensitive to temperature variations during aging, especially in the lower temperature range. The gel's viscoelastic behavior indicates changes with aging temperature, probably because of variations in the mobility of the pectin chains and, consequently, in the lifetime of the junction zones (Dasilva and Goncalves, 1994). HMP–sugar gels are formed by a combination of hydrogen bonding and hydrophobic effects. Because magnitudes of the latter are affected by the solute used and temperature, gel strength and rate of structure development are also affected. Technologically important events that take place during the sol–gel transition have been considered, including a profile of complex viscosity during gelation, and the effects of rate of cooling and pectin concentration (Dasilva and Rao, 1995). Structure developments in HMP–fructose gels were also characterized by Rao and Cooley (1993, 1994). Weaker pectin networks are formed under thermal conditions unfavorable to the development of hydrophobic interactions. Gelling time and elastic modulus have a complex dependence on temperature, which can be attributed to the different thermal behaviors of the intermolecular interactions that stabilize the non-permanent cross-links of

these physical networks. The influence of temperature on the dynamic and steady-shear rheology of pectin dispersions was studied by Dasilva *et al.* (1994). The authors used the time–temperature superposition principle to calculate activation energies, and their dependence on temperature and shear rate was analysed.

Low-ester pectin gels do not require a high solids content or low pH, but they do need the presence of calcium, which can be provided by the fruit pulp if a fruit product is desired. Calcium binding to low-ester pectin cannot be explained as a simple electrostatic interaction: it involves intermolecular chelate binding of the cation leading to the formation of macromolecular aggregates (Kohn and Luknar, 1977). An 'egg-box' model has been suggested for primary junction zones in the low-ester pectin molecular gel network (Rees, 1982). Chain segments with 14 or more residues having a ribbon-like symmetry are believed to form parallel-oriented aggregates. Chelate bonds with oxygen atoms from both galacturonan chains formed by calcium ions are formed when calcium ions fit into 'cavities' in the structure (Fig. 5.6). Although they differ from those involving high-ester gels, the concepts of good manufacturing practice need to be maintained, and the ingredients appropriately selected. Main differences between high- and low-ester systems are the ability to melt a low-ester pectin gel and the immediacy with which solidification occurs in the low-ester pectin system, relative to the slow rate of the high-ester pectin gel. Amidated low-ester pectins are usually able to jellify preserves, jams and jellies with calcium ions originating from fruit and water (Broomfield, 1988). Non-amidated low-ester pectins generally require a higher calcium level and the addition of extra calcium is very often necessary to obtain proper gel formation (Christensen, 1986). The degree of amidation and esterification controls the readiness of low-ester pectin reactions with calcium to induce gel formation. Low-ester pectins with a DE of 25–35% (non-amidated), and pectins with 20–30% DE and 18–25% DA are highly reactive with calcium and are, therefore, used in low-calcium and low-soluble-solids content systems. Pectins with a low ester content of 35 to 45% (non-amidated) and those with 30–40% DE and

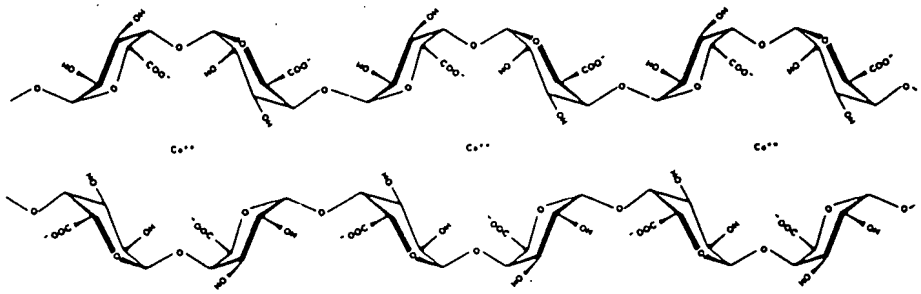


Figure 5.6 Junction zone in a low-ester pectin gel.

10–18% DA, because of their lower calcium reactivity, can serve in high-calcium or high-soluble-solids content systems (Buhl, 1990).

5.8.1 Gel properties

Results of gel-strength measurements yielded by different methodologies are not necessarily correlated. This is partly the result of the different properties defined and of the different measures such as deformation within the elastic limits and the force needed to break the gel. Traditional methods are the SAG method (this traditional method, based on Cox and Higby's work (1944) and adopted by the Institute of Food Technologists (1959), defines a jelly grade designation of 150° USA-SAG as the transformation of 150 parts sucrose by 1 part pectin into a gel of 65° Brix, pH 2.2–2.4 and a gel strength of 23.5% SAG after being cast for 2 min and removed from a standard glass with exactly specified inner dimensions), the use of the LFRA texture analyser, the Boucher Electronic Jelly Tester, the FIRA tester, the family of universal testing machines, the Herbstreith pectinometer, a spreadameter and the Bostwick Consistometer. In addition to the variety and plurality of gel texture measurements, other measurements of time–temperature relationships have also been developed within the industry. Gelation is influenced by the degree of methyl esterification, amidation, pectin concentration, water activity, the presence of calcium ions and pH.

The profile of shear modulus temperature meltdown of pectin gels was described by Clark *et al.* (1994). In this study, a cascade-theory approach to biopolymer gelation was developed to describe variations in the shear modulus with temperature for thermoreversible gels. The broadness of this 'melting transition' is seen to depend critically on the enthalpy of cross-linking, whereas the critical gel-melting temperature is determined by additional factors, such as the entropy of cross-linking, polymer concentration, molecular weight and the number of cross-linking sites. When the model was used to fit experimental data from a pectin system, a broad melting transition and the high melting point of the pectin system were consistent with much smaller negative values for these parameters (Clark *et al.*, 1994).

The mechanisms of gelation hint at a combination of hydrogen bonding and hydrophobic interactions in the case of high-ester pectin gel formation. The hydrophobic parts of the high-ester pectin molecule are the ester groups. Contact between these hydrophobic areas is associated with energy contribution. Hydrogen bonds formed between adjacent galacturonan chains contribute even more to decreases in the energy of junction-zone formation. However, the energy contribution of the hydrophobic interaction is necessary in order to make the sum of the energy contributions favoring gelation large enough to exceed energy contributions that resist gelation. The proposed mechanism suggests that gel formation with high-ester pectins

relates to the rigidity of its molecule, its correlation to DE level and the presence of sugars in the system. For low-ester pectin, the 'egg-box' model used to explain alginate gelation is proposed. Twofold helices are bridged by calcium ions of opposing carboxyl groups. In dried pectins, helices with three subunits per turn are detected, hinting that helix structure changes from twofold to threefold when the gel is dried to a powder. Support for the 'egg-box' model also comes from the direction of equilibrium dialysis: ~50% of the calcium ions cannot be removed by exposure to a very large concentration of univalent cations.

5.9 Applications

The most common use of pectins is in the preparation of jams, jellies, or similar gels. Detailed information on jam production can be found elsewhere (Kertesz, 1951). Ordinary jam is generally made from high-ester pectin, whereas the low-ester pectins are used when a softer, more spreadable texture is desired. If fruit particles (pulp) are to be contained in the jam, a high gelation temperature is used and solidification begins almost immediately after filling the containers, with almost no floatation of the particles being observed. If very large containers are used for jam-filling, then pectins with lower filling temperatures should be considered, to minimize flavor and color destruction, especially in the center of the container. The rheological indices of fruit content in jams and the effect of formulation on flow plasticity of sheared strawberry and peach jams has been studied (Costel *et al.*, 1993). The effect of formulation factors on Casson yield values measured at low and medium shear rates are reported.

To prepare low-sugar (less sweet) gums, low-ester pectins are used in combination with calcium in an amount related to gelation temperature and the quality of the formed texture. When jellies that contain no particles are produced, slow-setting pectins that solidify a long time after filling, allowing air bubbles to float and escape from the product, are preferred. For confections, a slow-setting high-ester pectin is used. The solid content of such preparations is high, ~78%, in contrast to ordinary jams at ~65% or low-sugar jams at ~30–55% (Rolin and De Vries, 1990). For baked goods, a heat-resistant gel is usually produced with a soluble-solids content of 45–75% and, depending on the type of pectin used, a typical dosage yields pH values of 3.3–3.6; if a cold-setting gel is used, a product with ~61% soluble solids and pH 4.0 is produced using rapid-set high-methyl-ester pectin (0.7%). Heat-resistant gels are generally prepared from high-ester pectins but can be produced from low-ester pectins if calcium citrate is used in the formulation to elevate the gelation temperature subsequent to the setting of the system. Fruit preparations for dairy products are often sold as semi-gel/thixotropic products with a typical soluble-solids content of 30–

65% and a pH of 3.6–4.0, usually prepared with 0.3–0.6% low-methyl-ester pectin. These should be prepared in such a way that the big fruit chunks or berries are distributed uniformly even after storage and pumping or transport (Rolin and De Vries, 1990).

Pectins are used to prepare bakery fillings and glazes. Oven-resistant high-sugar jams are produced at a solids content of ~70% using rapid-set pectin. Another demand of such products is mechanical stability. The less the gel is ruptured, the lower the syneresis at elevated heating temperatures. Non-amidated low-ester pectins are recommended for the production of bakery jams with satisfactory stability. Low-ester pectin gels are produced with ~65% soluble solids and a relatively high dosage of calcium-reactive, low-ester pectin. Before being applied to the baked goods, water is added, then the gel is heated to ~85°C to induce melting, and hot coating of the product follows. Upon cooling, a glossy coverage is formed. Other studies on the physical and mechanical properties of highly plasticized pectin–starch films can be found (Coffin and Fishman, 1994).

The thermomechanical properties of pectin and polyvinyl alcohol (PVA) blends have been recently studied (Coffin *et al.*, 1996). Increasing the amount of PVA in the blends reduced the storage and loss modulus of the films above the glass transition temperature. Changes in the molecular weight and degree of ester hydrolysis of PVA exerted a rather small effect on the blends. The composition should be targeted to the specific aim (Coffin *et al.*, 1996).

Stabilization of pasteurized or sterilized, acidified milk products (pH values of ~3.5–4.2) can be achieved by using high-ester pectins with DE greater than ~70. Acidification can be produced by either fermentation or the addition of fruit juice. If casein stabilization is not achieved, an undesirable grain-like texture is obtained. The pectin, added before homogenization, is absorbed onto the casein particles, which have a positive charge in the unstabilized milk. If the amount of added pectin is small, then the charge is neutralized and the system tends to collapse owing to the removal of repulsive forces. If pectin addition is continued however, a new repulsive force builds up, resulting in stabilization of the acidified milk system. Hydrophobic as well as electrostatic interactions are important in stabilizing pectin–casein dispersions (Pereyra *et al.*, 1995). The shear rate and time dependency of stirred yoghurt rheology were evaluated as influenced by added pectin and strawberry concentrate (Basak and Ramaswamy, 1994). The rheology of the flavored yoghurt was influenced by both pectin (0–0.5%) and the concentrate, and the desired product viscosity could be obtained by postfermentation mixing of stirred yoghurt with the pectin and fruit concentrate. Quality requirements for yoghurt–fruit preparations and the rheological parameters used to assess their properties are discussed in Kratz and Dengler (1995). Three different types of pectin were compared in yoghurt preparations. The possibility of using yoghurt–fruit

preparations instead of increasing the dry matter content in order to improve the consistency of fruit yoghurt was considered. In fat-free yoghurt, good mouthfeel and stability were achieved by increasing the percentage of milk solids and adding a mixture of gelatin, starch and pectin (Moller, 1995). In order to eliminate unacceptable viscosity, poor mouthfeel and syneresis in pasteurized yoghurt, processing conditions need to be adjusted and starch, gelatin and pectin added (Moller, 1995). The use of non-traditional additives (dried fruit and vegetable powders) in the manufacture of cultured milk products for therapeutic and prophylactic uses was reported by Arkhipova and Krasnikova (1994). Results of clinical trials showed that in less than 2 weeks patients receiving these cultured milks shared increased appetites, improved intestinal microflora and 20–25% decreases in blood cholesterol level (Arkhipova and Krasnikova, 1994).

Pectin is also used to stabilize clouding in beverages. Such stability is dependent on the nature and amount of the pectin present. Natural clouding agents can be produced (Elshamei and Elzoghbi, 1994) from orange and lemon peels using enzyme preparations to hydrolyse the pectin in the peel. The chemical and physical properties of the clouds were evaluated in parallel to the drink's properties, taste and stability. The cloudiness of the produced drinks stabilized after 42 days of storage at 25°C. Other reports on the physicochemical nature of pectin associated with commercial orange juice clouding can be found elsewhere (Klavons *et al.*, 1994).

The confectionery industry makes use of slow-set high-ester pectins to prepare fruit jellies and jelly centers. Low-ester pectins are also used to impart thixotropic behavior at low concentrations, or to achieve a cold-set type of gelation if diffusion of calcium ions occurs. The use of pectin in confections permits the manufacture of products with tailor-made textural properties, good flavor release and compatibility with continuous processing. High-methoxy pectin was also reported to produce coatings that inhibit lipid migration in a confectionery product (Brake and Fennema, 1993).

Low-ester pectins are used as gelling agents and texturizers in many very different food products such as artificial caviar, meat products and dessert jellies. Pectin–alginate combinations have a synergistic effect in terms of gel-formation properties. Xanthan and pectin together can serve as an appropriate stabilizer for salad dressings. The incorporation of pectin in water-ice and sherbet preparations improves product acceptability by minimizing the growth of ice crystals. Galactomannans in combination with pectin serve to stabilize ice cream. Pectins are used to stabilize emulsions. Modified pectins in whey–protein emulsions (Einhornstoll *et al.*, 1996) were found to stabilize the whey protein at high enough concentrations. For any individual utilization, the most suitable pectin needs to be selected. Frozen fruit preparations are improved by incorporating pectin into the product. Pectins can be used for coating, in recipes of spray-dried instant tea and for many other products.

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Pectins are mixtures of polysaccharides that originate from plants, contain pectinic acids as major components, are water soluble, and are able to form gels under suitable conditions (See section on Physical Properties).

In this chapter, as is frequently done, the term pectin will be used in a generic sense to designate those water-soluble galacturonoglycans of varying methyl ester content and degree of neutralization that are capable of forming gels under suitable conditions (See section on Physical Properties), i.e., other polysaccharides that may be present in commercial mixtures will be ignored.

Pectins are subdivided according to their degree of esterification (DE), a designation of the percent of carboxyl groups esterified with methanol. Pectins with DE > 50% are high-methoxyl pectins (HM-pectins); those with DE < 50% are low-methoxyl pectins (LM-pectins).

The degree of amidation (DA) indicates the percent of carboxyl groups in the amide form (See section on Chemical Properties).

Structure

For a more detailed discussion of the chemical structure of pectins, see reference 3.

Pectin, a structural, cell-wall polysaccharide of all higher plants, like most other polysaccharides, is both polymolecular and polydisperse, i.e., it is heterogeneous with respect to both chemical structure and molecular weight (4). From molecule to molecule, in any sample of pectin, both the number and percentage of individual monomeric unit types will vary, and the average composition and distribution of molecular weights can vary with the source, the conditions used for isolation, and any subsequent treatments. Because both parameters determine physical properties, various functional types of pectin can be produced by controlling the source, isolation procedure, and subsequent treatment(s)

Pectin is primarily a polymer of D-galacturonic acid. The principal and key feature of all pectin molecules is a linear chain of (1→4)-linked α-D-galactopyranosyluronic acid units, making it an α-D-galacturonan [a poly(α-D-galactopyranosyluronic acid) or an α-D-galacturonoglycan].

In all natural pectins, some of the carboxyl groups are in the methyl ester form. Depending on the isolation conditions, the remaining free carboxylic acid groups may be partly or fully neutralized, i.e., partly or fully present as sodium, potassium or ammonium carboxylate groups. The ratio of esterified D-galacturonic acid units to total D-galacturonic acid units is called the degree of esterification (DE) and strongly influences the solubility, gel forming ability, conditions required for gelation, gelling temperature, and gel properties of the preparation.

In pectin from some sources, some of the units occur as O-2 or O-3 acetates. Such esterification hampers gelation, so much so that complete inhibition of gelation occurs when one out of eight

of counterions in the solution. For example, addition of monovalent cations effects a reduction in viscosity, the degree of which is greater with decreasing DE. Addition of salts of di- and trivalent cations has an opposite effect (20). In general, viscosity, solubility, and gelation are related, i.e., factors that increase gel strength, for example, will increase the tendency to gel, decrease solubility, and increase viscosity, and vice versa.

These physical properties of pectins are a function of their structure which is that of a linear polyanion (polycarboxylate). As such, monovalent cation salts of pectins are highly ionized in solution, and the distribution of ionic charges along the molecule tends to keep it in an extended form by reason of coulombic repulsion (21). Furthermore, this same columbic repulsion between the carboxylate anions prevents aggregation of the polymer chains. (The number of negative charges is, of course, determined by the DE.) In addition, each polysaccharide chain, and especially each carboxylate group, will be highly hydrated. Solutions of monovalent salts of pectins exhibit stable viscosity because each polymer chain is hydrated, extended, and independent.

Because the commercial importance of pectin is predominately the result of its unique ability to form spreadable gels in the presence of a dehydrating agent (sugar) at a pH at or near 3 or in the presence of calcium ion (jams, jellies, and marmalades made from fruit juices or whole fruit), that is the property most often studied and focused upon. Factors that determine whether gelation can occur and that influence gel characteristics are pH, concentration of cosolutes (sugars), concentration and type of cations, temperature, and pectin concentration. The ways in which these factors influence gelation are dependent upon the following molecular properties of the specific pectin: molecular weight (4), degree of esterification (DE), degree of amidation (DA), presence of acetate esters, and heterogeneity. All these parameters are interdependent. In general, under similar conditions, the degree of gelation, the gelling temperature, and the gel strength are generally proportional to each other and each property is generally proportional to the molecular weight and inversely proportional to the DE.

As the pH is lowered, i.e., as the hydrogen ion concentration of the solution is increased, ionization of the carboxylate groups is repressed, i.e., the highly hydrated carboxylate groups are converted into only slightly hydrated carboxylic acid groups. As a result of losing some of their charge, the polysaccharide molecules no longer repel each other over their entire length; and as a result of losing some of the water of hydration, they can associate over a portion of their length to form a gel. Apparent pK values (pH at 50% dissociation) vary with the DE of the pectin (22); a 65% DE pectin has an apparent pK of 3.55, while a 0% DE pectic acid has an apparent pK of 4.10. However, pectins with increasingly greater degrees of methylation will gel at somewhat higher pH, undoubtedly because they have fewer carboxylate anions at any given pH (See later paragraph). pH affects gel texture more than gel strength.

Exhibit C

Table I. Pectin and Unavailable Carbohydrate Content of Fruits, Nuts, and Vegetables

	Dry matter	Pectin		Unavailable carbohydrate
		Ca Pectate	Carbazol	
	Fresh weight of edible portion (%)			
Fruit				
Apple	15.9	0.78	0.45	1.7
Apricot	13.4	1.00	0.70	2.1
Banana	29.3	0.94		3.4
Blackberry	18.0	0.94	0.30	7.3
Blueberry	16.8		0.30	
Cherry	18.5	0.39	0.36	1.7
Fig	15.4	1.11		2.5
Grape	19.3	0.19	0.20	0.4
Grapefruit	9.3	3.90		0.6
Lemon	14.8	2.90		5.2
Loganberry	15.0	0.59		6.2
Orange	13.9	2.36		2.0
Peach	13.8	0.39	0.64	1.4
Pear	17.0	0.49	0.46	2.5
Pineapple	15.7	0.09		1.2
Plum	15.9	0.44	0.59	2.1
Raspberry	16.8	0.97	0.34	7.4
Rhubarb	5.8	0.44	0.34	2.6
Strawberry	11.1	0.75	0.50	2.2
Watermelon	7.4	0.18		
Nuts				
Peanut	95.5	5.98		8.1
Walnut	76.5	5.80		5.2
Vegetable				
Asparagus	7.6		0.22	1.5
Avocado	7.8	2.86		
Bean	8.4	0.70	0.55	3.0
Beet	12.9	0.91	0.42	3.1
Broccoli	9.2		0.49	4.2
Brussels Sprout	9.2		0.78	4.8
Cabbage, red	10.3		0.53	3.4
Carrot	10.2	2.00	0.96	2.9
Cauliflower	10.9		0.38	1.5
Cucumber	3.6	0.16	0.17	0.4
Eggplant	6.6	0.47		2.5
Garlic	38.7	1.11		
Kohlrabi	9.7		0.38	
Lettuce	4.8		0.34	1.4
Okra	11.1	1.53		
Onion	7.2	0.35	0.44	1.3
Pea	21.5		0.34	5.2
Pea, with pod		0.57		
Pepper, green	16.6	0.09		
Potato	24.2	0.83	0.34	2.1
Pumpkin	5.3	1.24	0.20	0.5
Radish	6.7		0.45	1.0
Rutabaga	13.0		0.80	
Soybean	90.0	3.45		
Spinach	14.9		0.33	6.3
Sweet potato	28.0	0.78		2.1
Tomato	6.6	0.20	0.30	1.5
Turnip	6.7	0.29		2.8
Yam	26.5	0.62		

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Exhibit D

pectic acid, and pectinic acid all occur in the solid state (fibers) as right-handed (3_1) helices with a three-fold screw axis (trisaccharide repeat) (12-15). In solid pectinic acid, the polymer molecules pack so that the chains are parallel to each other; the pectates pack as corrugated sheets of antiparallel chains (14,15).

It is further suggested that junction zones in pectinic acid (HM-pectin plus sucrose) gels are formed by a columnar stacking of methyl ester groups to form cylindrical hydrophobic areas parallel to the helix axes. Two models for the formation of junction zones in calcium pectate (LM-pectin) gels have been proposed. One suggests an aggregation of chains by a crosslinking of carboxylate anions with calcium ions to form a structure similar to that of the corrugated sheets of antiparallel helices (3-6 chains in an average junction zone) found in solid calcium pectate (15). The other is the "egg box" model used to describe the formation of calcium alginate gels (16,17). This model is proposed because of the close similarity between (1→4)-linked poly(α-D-galacto-pyranosyluronic acid) segments of pectic acid and (1→4)-linked poly(α-L-gulo-pyranosyluronic acid) segments of alginic acids, segments that are mirror images except for the configuration at C-3. From circular dichroism and equilibrium dialysis studies, it has been concluded that interchain association of hydrated pectinic acid molecules, in the presence of swamping levels of monovalent counterions, is limited to the formation of dimers of chains of 2_1 helical symmetry with specific site-binding of calcium ions along one face of each participating chain (18,19; see also reference 20). When Ca^{2+} is the sole or principal counterion, these dimers further aggregate without rearrangement, leading to an approximate doubling of the amount of Ca^{2+} bound cooperatively (18,19). Based on available information, the Unilever Research group (19) has concluded that drying of a calcium pectinate gel effects a polymorphic phase transition in which associated, regular, buckled chains with two-fold symmetry ("egg box") as found in L-guluronoglycan chain segments are converted into associated chains with three-fold symmetry as found in solid state calcium pectinate (12-15). It should be noted that the axial-axial linkages in a chain of aldohexopyranosyl units linked 1→4 gives a buckled conformation naturally (Figure 1) and that the gel structure(s) is(are) as yet not well understood.

Physical Properties

Pectins are soluble in pure water, but they are insoluble in aqueous solutions in which they would gel at the same temperature if dissolved at a higher temperature. Monovalent cation (alkali metal) salts of pectinic and pectic acids are usually soluble in water; di- and trivalent cation salts are weakly soluble or insoluble.

Although pectins are not employed as thickening agents, pectin solutions exhibit the non-Newtonian, pseudoplastic behavior characteristic of most polysaccharides. As with solubility, the viscosity of a pectin solution is related to the molecular weight, DE, and concentration of the preparation and the pH and presence

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Amidation results in a higher gelling temperature and a decreased need for a divalent cation.

The distribution of carboxyl/carboxylate groups also affects gelation. Pectins with blocks of methyl ester and carboxyl groups (as opposed to a random distribution) generally produce weaker gels and have a greater requirement for divalent cations.

Chemical Properties

Dissolved pectins undergo deesterification and depolymerization in aqueous systems. The pH of greatest stability is about 4. At pH values both above and below 4, deesterification and depolymerization occur concurrently, with the rate of deesterification being greater than the rate of depolymerization. The presence of solutes, which lowers water activity, reduces the rates of both reactions.

Deesterification occurs by normal acid- and base-catalyzed mechanisms of ester hydrolysis. Depolymerization at low pH values occurs by means of acid-catalyzed hydrolysis of glycosidic bonds (24). Acid-catalyzed hydrolysis occurs preferentially at the L-rhamnopyranosyl glycosidic bonds. Hydrolysis of these linkages produces galacturonoglycan chains with a degree of polymerization of about 25 (6,9). Side chains, particularly those containing L-arabinofuranosyl units, should also be preferentially removed by hydrolysis because of the inherent stability to acid-catalyzed hydrolysis of glycuronosyl glycosidic bonds and the inherent lability of furanosyl glycosidic bonds (24). However, if the side chains are attached to rhamnogalacturonan sequences (8), it should not be possible to convert "hairy" regions to "smooth" regions by treatment with acid because the lability of the L-rhamnopyranosyl bonds would result in concurrent depolymerization of the main chain.

At pH values of 5-6 pectin solutions are stable only at room temperature. As the temperature is raised, pectin chains cleave by a beta-elimination reaction (25-39) (Figure 1), a reaction which is stimulated by organic anions (40). Deesterification of pectin proceeds simultaneously with the beta-elimination depolymerization reaction, which occurs only at monosaccharide units that are esterified. At pH values above 6, deesterification and depolymerization are rapid reactions even at room temperature, the rate of each reaction increasing with increasing pH.

Hydroxyl-group reactions, such as etherification, acetalation (41), esterification (42-51), and oxidation, can be done in the same manner as they are on other polysaccharides. Esterifications (47) of carboxyl groups and interactions with cations, including polycations such as proteins below their isoelectric pH, occur as they do with other glycuronoglycans. Reduction of carboxyl groups to hydroxymethyl groups has been done with diborane (52, see also 53) and by borohydride treatment of methyl and hydroxyethyl esters (54). Reduction of carboxyl groups which have been activated with a water-soluble carbodiimide should be straightforward (55,56).

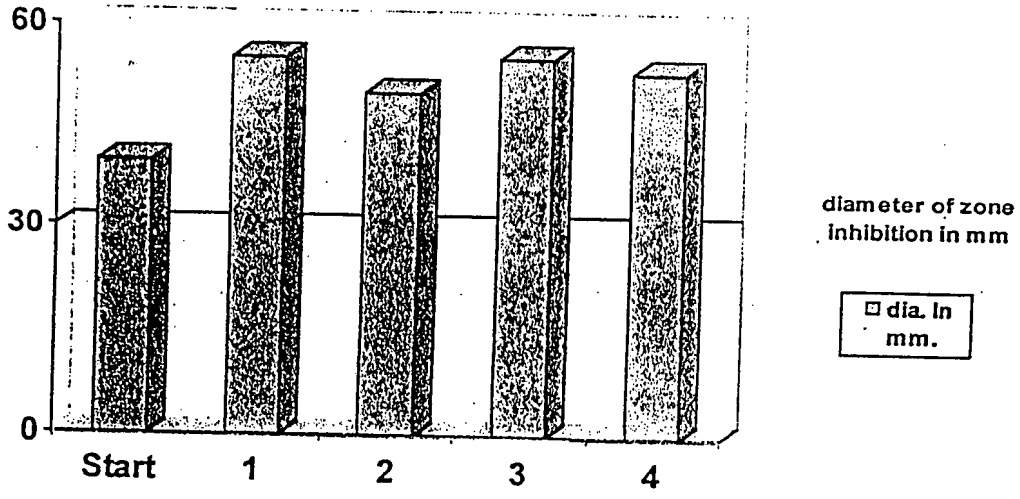
When ammonia (57-61) is used to prepare LM-pectin from HM-pectin, some of the methyl carboxylate groups are converted into carboxamide groups, producing "amidated pectin". The

TABLE 1

In-Vitro Reusability of Silver Alginate (**ALGIDEX AG**)

Materials & Methods:

A one (1) square inch piece of silver alginate dressing (ALGIDEX AG) was checked for reusability against *Ps. Aeruginosa.* Nutrient agar in which, the agar content was increased to 10% was inoculated with 0.1 ml of actively growing culture and surface spread. Hard agar was used to prevent too rapid water uptake from the gel. The Algidex Ag was placed on the agar surface and diffusion was allowed to commerce for 3 hours in refrigeration. The plate was then incubated at 37° Celsius for 16 hours. The zone of inhibition in mm was measured and the gel was then transferred to another inoculated agar plate. This process was repeated 4 times.



Silver-Coated Dressing Acticoat Caused Raised Liver Enzymes and Argyria-like Symptoms in Burn Patient

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Background: Treatment of acute burn wounds with silver sulfadiazine (SSD) has raised concern about potential silver toxicity. Numerous adverse reactions and side effects have been reported and an increasing resistance to SSD, especially in Pseudomonas strains, have motivated researchers to search for an alternative wound dressing.

Methods: Recently, a silver-coated wound dressing Acticoat (Smith & Nephew, Inc.) has become available for use in burn patients. It is a three-ply dressing, consisting of an inner rayon/polyester absorptive core between two layers of silver-coated, high-density polyethylene mesh. In a moist environment, the nanocrystals of silver are released and improve the microbial control in the wound.

Results: After 1 week of local treatment with Acticoat in a young, previously healthy 17-year-old boy with 30% mixed depth burns, hepatotoxicity and argyria-like symptoms, a grayish discoloration of the patient's face, appeared. The silver levels in plasma (107 μg/kg) and urine (28 μg/kg) were clearly elevated, as well as the liver enzymes. As soon as the local application of Acticoat was aborted, the clinical symptoms and liver enzymes returned to the normal values.

Conclusions: This is the first report on silver toxicity in a patient with 30% burns who received Acticoat for local treatment. Due to substantial experiences with adverse SSD reactions and side effects, it is appropriate to keep the possibility of a toxic silver effect in burn patients treated with Acticoat silver-coated wound dressing in mind. The silver levels in plasma and/or urine should be monitored.

Key Words: Burns, Acticoat, Silver toxicity.

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The destruction of a large area of the skin is a life-threatening condition due to the loss of essential body fluids and possible occurrence of wound infection, which may lead to septicemia. The core events in the management of large, deep burns are wound excision and closure.[1] In superficial burns and smaller mixed-depth thermal injuries, one usually has time to allow the wound to fully evolve to a point where it is easy to determine burn depth on physical examination. Therefore, safe coverage of the wound surface is necessary during that time. The aims of topical antimicrobial therapy have been to delay the growth of the patient's endogenous bacteria and to prevent the colonization of the dressed wound by external nosocomial organisms, while promoting the healing of the wound and minimizing the desiccation, pain and discomfort associated with dressing care.

The ideal skin substitute should be inexpensive; have a long shelf life; be usable off the shelf; be nonantigenic, durable, flexible, and prevent water loss; be a bacterial barrier, drape well, be easy to secure, grow with the child, and applicable in one operation; should not become hypertrophic; and—it does not exist yet.[2]

A variety of different factors have been proposed as having a negative impact on the speed of wound healing. One of the major contributors to delayed wound healing is a prolonged inflammatory response in the wound. Normally, the inflammatory response occurs following wounding and is initiated to help the body clean up tissue debris, counter any invading microorganisms, and signal the appearance of cells required for the synthesis of new tissue components. A prolonged inflammatory response may result in the destruction of tissue through the same mechanisms that normally have protective and restorative functions. The presence of bacterial cells within the wound is known to amplify and perpetuate the local inflammatory response. However, the presence of a high level of bacterial cells causes the prolonged inflammatory response in the absence of appropriate clinical measures to reduce the bacterial burden in the wound.[3]

Silver compounds are widely used as an effective antimicrobial agent to combat pathogens (bacteria, viruses, and eukaryotic microorganisms) in the clinic and for public health hygiene. Silver ions (Ag^+) are microcidal at low concentrations and have been used for decades as a topical antimicrobial agent for burns treatment. Elemental silver requires ionization for antimicrobial efficacy. It has a broad range of antimicrobial actions, including the disruption of the bacterial cell wall structure, the disruption of key bacterial enzymes such as cytochrome b and a_3, and interaction with the nucleic

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Exhibit H

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acids caused by its preferential binding to nitrogenous groups in guaninen and other nucleotides.[4]

CASE REPORT

While at work, a highly flammable solvent drenched the trousers of a 17-year-old apprentice boy. When he pulled a cigarette lighter out of his pocket, his trousers caught fire. He undressed immediately and cooled the wounds in the shower. The previously healthy young man sustained 30% total burn surface area (TBSA) superficial and deep dermal flame burns to his legs, buttocks, and hands. After initial resuscitation and analgesia at the local hospital, the patient was transferred to our burns unit 2 hours postinjury. On admission, he was in a stable clinical condition and on a Ringer-Lactate drip. He was 170 cm tall and weighed 61 kg. In the anamnesis, it was stated that he was allergic to wasp and bee venom, smoked moderately, and occasionally drank alcohol.

On admission, the patient was brought to the operating room for further wound evaluation and treatment. His body temperature was 36.3°C; he was conscious and pain free. The initial assessment of the buttocks and hands was that of partial thickness burns, and the leg burns were assessed to be deeper dermal burns that might require grafting. In anesthesia, the patient was washed completely, his wounds cleansed, and any loose or devitalized tissue were removed and rinsed with sterile water. The Acticoat (Smith and Nephew Inc.) wound-dressing was moistened with sterile water and applied to the complete burn area, followed by wet and dry sterile gauze dressing and secured in place with elastic netting. Blood samples were taken. Later on, the patient's body temperature was well maintained; he felt comfortable and was in a good mood. With additional intravenous fluid therapy, his vital parameters were stable and diuresis was adequate. Soon he regained his appetite and intravenous resuscitation was finished 24 hours later.

The Acticoat dressing was kept moist with distilled water and changed on day 4 postinjury. The wounds were checked, swabs taken, and, after cleansing, the Acticoat was reapplied. Macroscopically there were no symptoms of any local infection, no substantial exudate was present, but some clear appearing gel covered the wound in a few areas. The patient did not show any symptoms of septicemia and was doing well.

On day 6 postinjury, Acticoat was changed for the second time and the wound showed good healing progress without local infection.

At the same time, the patient gradually developed a grayish discoloration of his face with remarkable pale-bluish lips, but was not cyanotic. He appeared ill and complained about being tired and having no appetite; simultaneously he was found to have elevated aspartate aminotransferase, alanine aminotransferase, and gamma-galactosyl transferase liver enzymes. Total bilirubin, lactate dehydrogenase, and cholinesterase remained within the normal range. With the exception of low-dose paracetamol, the patient had not received any potentially hepatotoxic drugs. Serum methemoglobinemia was negative as well.

An abdominal ultrasound showed an only slightly enlarged liver and spleen with no focal abnormalities; the intestine, pancreas, gallbladder, and urogenital system were also without pathologic findings. His renal function was always normal.

Screenings for hepatitis A, B, and C viruses, as well as cytomegalovirus and Epstein-Barr virus, were negative. Wound culture swabs remained sterile or showed a growth of fecal strains in the inguinal burn wound (*Escherichia coli, Enterococcus* sp.) and moderate *S. aures* colonization on one knee.

The patient's general condition, laboratory results, and skin discoloration did not improve. On day 7 postburn, the blood and urine silver levels were checked and found to be profoundly elevated. Except Acticoat, no other source of silver was used on or by this patient. The Acticoat dressing was removed the same day and not used again. The local wound treatment was changed to a betadine (BI) ointment dressing.

In the following days, the dressings were changed at regular intervals. Initially with the Acticoat treatment, the wound had shown good healing progress, but after the local therapy regime had been changed, the healing progress had been disrupted. Simultaneously, the discoloration of the face gradually faded over a period of days and the liver function tests were returning to their normal values. On day 17 postinjury, when his clinical condition had changed for the better, 8% of the TBSA was excised and grafted. The take rate was satisfactory.

Finally, the patient made a full recovery and was discharged with still markedly elevated blood silver levels, 7 weeks postinjury. Ten months later silver was hardly detectable, neither in blood (0.9 μg/kg), nor in urine (0.4 μg/kg). Table 1 shows laboratory checks and local wound treatment.

DISCUSSION

There is much published evidence to show that silver is a potent biocide and the idea of including silver ions in dressings to combat or prevent local infection is attractive.

A 0.5% silver nitrate solution is the lowest concentration that remains active against bacteria in vitro and in vivo in burns and has no toxic effect on growing epidermal cells. $AgNO_3$ is active against *S. aureus*, hemolytic streptococci, and generally against *Pseudomonas aerugenosa* and *E. coli*. The main complication occurring during the treatment is a drop in serum sodium and chloride, due to precipitation between Na^+, Ag^+, and Cl^-, HCO_3^-, CO_3^- and protein anions, which leads to the production of very slightly soluble or insoluble salt solutions. In general, it was assumed that silver is not taken up by the body, but in postmortem examinations elevated silver levels in the kidney, spleen, liver, and muscles have been found.[5]

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Table 1 Laboratory Checks and Local Wound Treatment

	Reference Range	Day														
		1	4	7	8	9	10	11	12	15	16	17	22	27	49	97
WBC (5,550–14,000)/μL		13.2	8.3	10.1	12.1		13.7	13.7	12.2		9.6			6		
Hemoglobin (11.5–13.5) (g/dL)		15.7	14	13.4	14.1		13.6	13.5	13.4		13.7			12.1		
Platelet count (140,000–440,000)/μL		162,000	188,000	304,000	353,000		428,000	448,000	433,000		437,000			299,000		
Blood urea (10–45) (mg/dL)		15	26	22	18		22	21	20		18			23		
Serum creatinine (0.50–1.30) (mg/dL)		0.9	1	0.9	0.8		0.8	0.8	0.9		0.8			0.7		
Aspartate aminotransferase (−21) (U/l)		10	42	78	64		39	39	26		28			13	9	
Alanine aminotransferase (−22) (U/l)		8	69	233	193		143	134	111		90			45	11	
Gamma-galactosyl transferase (−19) (U/l)		8	24	94	96		78	71	63		48			29	14	
C-reactive protein (−8)(mg/L)		37	128	93	66		27	16	14		5			5		
Silver in blood (<0.21 μg/kg)*				107		100	104	104	99	85			83	71	42	13.3
Silver in urine (<0.21 μg/kg)*				28		15.1	13.6	10.3	6.8	9.8			4.5	3.8	2.3	1.5
Local treatment		Acticoat	Acticoat	Acticoat	BI	BI	BI	BI	BI	BI	BI	Surgury				

*Silver concentrations were determined with an Agilent 7500c inductively coupled plasma mass spectrometer (Agilent, Waldbronn, Germany) after microwave assisted mineralization of the serum and urine samples with nitric acid in an UltraCLAVE2 (EMLS, Leutkirch, Germany) at 250°C for 40 minutes.

The best known and the most widely used product is a topical cream that contains 1% silver sulfadiazine plus 0.2% chlorhexydine digluconate in a water immiscible cream base. The *Pseudomonas* strains nearly disappeared under the influence of silver sulfadiazine and the mortality in burn patients declined. Due to the absorption of silver from large bums and the possibility of silver toxicity occurring in some patients, the use of SSD came into question.[6–8]

Boosalis et al.[9] monitored plasma silver levels and urinary silver excretion in 23 patients with second- and third-degree burns who were treated with SSD. Mean serum concentrations were modestly elevated. In contrast to the serum levels, a high urinary excretion of silver was found, predominantly in patients with burns involving >60% TBSA, with peaks of 1,100 μg/24 hour (normal < 11 μg/24 hour). Coombs et al.[10] showed in their prospective study that plasma silver levels rapidly increased in bum patients treated with SSD. Twenty-two patients were studied; the age range was 17 to 80 years. Serum silver levels rose in all patients with burns >5% TBSA. The altered hepatic and renal laboratory results were not correlated with plasma silver levels.

SSD and silver, as an ingredient of the cream, have been linked to a variety of side effects and adverse reactions. The systemic effects range from allergic reactions[11] and erythema multiforme,[12] to the deterioration of the mental status[13] and frequent leukopenia. The drop in white blood cells (WBCs) occurs early in the course of the treatment and is not related to septic episodes. Withdrawal of SSD leads to a prompt recovery of the WBCs, but continuation of the SSD therapy does allow a slow WBC recovery as well. There is no increase in mortality with transient leukopenia.[14]

In addition to the reports on transient leucopenia[15–17] Gamelli et al.[18] showed in an in vitro study that SSD was directly cytotoxic to myelopoietic tissue and, in vivo, altered the myeloid cell compartment.

Due to a number of papers reporting side effects and adverse reactions to SSD, the high frequency of dressing changes and the fact that resistance to SSD, especially in *Pseudomonas* strains,[5] had been observed to arise, there was a demand for an alternative wound dressing for local burn wound treatment.

Recently, a silver-coated wound dressing, Acticoat has become available for use in burn patients. It contains a three ply-dressing, consisting of an inner rayon/polyester absorptive core, between two layers of a silver-coated high-density polyethylene mesh. In a moist environment, the silver nanocrystals (average crystal size of 15 nm) are released and improve microbial control on the wound. The silver coating in Acticoat consists of 0.2 to 0.3 mg silver per milligram of high density polyethylene, and is a binary alloy of silver (97%). It is less than 1 μm in thickness and engineered to change color in aqueous solution. The produced film coating is abrasion-resistant, nonadherent to the wound, and flexible. The extremely small size of the silver nanocrystals produces a very large surface area and the dressing core absorbs and

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accumulates moisture, thus maintaining a moist environment on the wound surface. The initial application is easy because it needs to be changed every 3 days (the antimicrobial barrier properties remain effective for a minimum of 3 days) and moistured once or twice a day with sterile water. In the instruction leaflet, there are only few contraindications and precautions listed.

Before human trials began, Acticoat was evaluated in vitro for the toxicity to mammalian tissue (minimal toxicity was found) for the release of silver in an aqueous environment (local concentration of silver ions 50 mg/L to 100 mg/L for up to 48 hours) and, in a porcine model, for adverse effects on wound healing (no adverse effects were detected). In these models, neutron activation analysis of plasma and urine revealed no evidence of systemic silver absorption with Acticoat.[19] In a clinical study, 30 burn patients were randomly treated with either 0.5% silver nitrate solution or Acticoat. The duration of the treatment was an average of 4 days. No adverse effects from use of Acticoat were found. The frequency of burn wound sepsis ($>10^5$ organisms/g tissue) was lower in the Acticoat group (5 vs. 16) and secondary bacteremia arising from infected burn wounds was also less frequent with Acticoat.[20] Plasma or urine samples for silver absorption were not obtained in this study. Yin et al.[21] tested the antimicrobial activity of Acticoat, 0.5% silver nitrate solution, 1% silver sulfadiazine cream, and 5% mafenid acetate solution on *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. Silver extracted from Acticoat had quite low minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against the tested bacteria, as had others, but Acticoat did kill the bacteria very quickly. In a controlled clinical study, Acticoat was tested as a donor site dressing and compared with Allevyn. Sixteen paired sites in 15 patients were evaluated. Acticoat-dressed donor sites took significantly longer for >90% re-epithelialization (14.5 ± 6.7 days vs. 9.1 ± 1.6), donor sites dressed with Acticoat had significantly worse scars at 1 and 2 months (the difference resolved by 3 months) and there was no difference in the incidence of positive bacterial cultures.[22]

We made a very similar observation in one 11-year-old boy whose donor sites were dressed with Acticoat. The wound healing was noticeably delayed (>2 weeks) compared with our current standard and it was difficult to remove Acticoat due to adherence to the wound ground on day 14 postsurgery. In contrast, Demling et al.[23] report an increased re-epithelialization of meshed skin grafts. In a porcine model of wound healing, a nanocrystalline silver-coated dressing promoted rapid metalloproteinase levels and enhanced cellular apoptosis.[3]

Particularly with regards to cultured skin substitutes, effective topical mixtures containing antimicrobial and antifungal agents are basic. For these reasons, alternatives have been investigated. Holder et al.[24] tested the antimicrobial activity of Acticoat, N-Terface, and Op-Site in strains of *S. aureus*, *P. aeruginosa*, *Candida albicans*, and divers gram-negative members of *Enterobactereicae*, isolated in the wounds of the burn patients. It was found that Acticoat has inherent antimicrobial properties, but, to be effective, hours of contact between Acticoat and the microorganisms are required. Acticoat also has a capacity to serve as an impenetrable barrier for all organisms tested in the study. Thomas et al.[25] also tested the antimicrobial activity of four silver-containing dressings (Acticoat, Actisorb Silver 220, Avance, and Contreet-H) against *S. aureus*, *E. coli*, and *C. albicans*. Acticoat produced the most rapid antimicrobial effect in vivo due to the rapid release of relatively large concentrations of highly active silver ions.

The antimicrobial efficacy of Acticoat wound dressing had been shown in several in vivo and in vitro studies. In contrast, there are few clinical studies published and no reports on adverse reactions or side effects with Acticoat therapy. We also failed to find any data on silver plasma and/or urine levels in humans treated with Acticoat.

Our patient was a young, healthy man who sustained 30% flame burns, 8% deep dermal. After 1 week of Acticoat treatment, his wounds were found to be healing rapidly and without pathologic findings. The patient never complained about discomfort or pain in the burn area. The first striking symptom was his facial appearance: a grayish discoloration and pale-bluish lips, gradually increasing. The evident argyria-like face discoloration in our patient was the final clue to perform a plasma and urine silver check. The phenomenon described does not match argyria, which is a permanent disorder caused by silver deposition in the skin's microvessels in patients who are exposed to chronic silver toxicity.[26,27]

CONCLUSION

In our case, it has to be assumed that the transient skin discoloration was related to the acutely elevated blood silver levels. The liver function abnormalities observed in our patient can only be an effect of acute silver toxicity because there was no other cause for liver damage; the patient was not taking drugs or alcohol and was not given potentially hepatotoxic medication. He was not dehydrated and had normal renal function at all times.

This evidentiary assumption that silver released from Acticoat was the cause for our patient's problems is supported by the fact that the clinical argyria-like symptoms disappeared and liver function tests returned to normal after the Acticoat local treatment was discontinued.

This is the first report on silver toxicity in a patient with 30% burns who received Acticoat for local treatment. Due to substantial experiences with adverse reactions and side effects to SSD, it is appropriate to keep the possibility of the toxic silver effect in burn patients treated with Acticoat silver-coated wound dressing in mind. The silver levels in plasma and/or urine should be monitored.

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An in vitro analysis of the antimicrobial properties of 10 silver-containing dressings

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The variation in the structure and properties of the many silver dressings now on the market might be expected to affect their antimicrobial properties. This study, which follows up a paper published last March, compares their performance

Silver-containing dressings, indicated primarily for the treatment or prevention of soft-tissue infections, depend on the ability of low concentrations of silver ions to kill a broad spectrum of microorganisms. Some of the new materials have been developed to carry and release silver in a controlled fashion. In others cases, a silver compound has been added to an existing product.

Given the considerable variation in the structure, composition and silver content of these new preparations, marked differences might also be anticipated in their ability to release silver in sufficiently high concentrations to exert a significant antimicrobial effect. This could have important implications for the dressings' clinical performance.

We have already described how the antimicrobial activity of four silver-containing dressings was compared in a laboratory-based study using three tests.[1] The dressings were: Acticoat (Smith and Nephew), Actisorb Silver 220 (Johnson and Johnson), Avance (SSL International) and Contreet-H (Coloplast).

This follow-up paper reports the results of further tests conducted in an identical manner on an additional six silver-containing dressings, together with a non-woven swab (negative control) and Acticoat (positive control).

Materials and methods

The six new dressings are described below, using the limited information provided by the manufacturers.

Arglaes (Medline)

Contains a mixture of an alginate powder and an inorganic polymer containing ionic silver. The alginate absorbs moisture to form a gel, and the silver complex breaks down in a controlled fashion to liberate ionic silver into the wound.

Aquacel Ag (ConvaTec)

Comprises a fleece of sodium carboxymethylcellulose (CMC) fibres containing 1.2% ionic silver. The dressing absorbs moisture to form a gel, binding sodium ions and releasing silver ions.

Calgitrol (Magnus Bio-Medical Technologies)

A silver alginate dressing comprising an absorbent foam sheet, one surface of which is coated with an alginate matrix containing ionic silver, together with a 'cleanser, moisturiser and a superabsorbent starch co-polymer'.

Contreet Ag (Coloplast)

A polyurethane foam dressing containing silver in a so-called 'hydroactivated' form, which is released as the foam absorbs liquid.

Silverlon (Argentum Medical)

A knitted fabric dressing, silver-plated by means of a proprietary autocatalytic electroless chemical (reduction-oxidation) plating technique. This coats the entire surface of each individual fibre, resulting in a very large surface area for the release of ionic silver.

Silvasorb (Medline)

Composed of a synthetic, polyacrylate, hydrophilic matrix in which microscopic silver-containing particles are

dispersed or suspended. On exposure to moisture, silver is released in a controlled fashion.

Test organisms

This study set out to compare the antimicrobial properties of the dressings rather than investigate the antimicrobial activity of silver itself.[1] Three standard organisms were therefore used:

- Gram-positive: *Staphyloccocus aureus* (ATCC 6538P)
- Gram-negative: *Escherichia coli* (ATCC 8739)
- A yeast: *Candida albicans* (ATCC 2091).

Test methods

Three different methods were designed to compare various aspects of performance. Full details are given in the original article.[1]

- **Zone of inhibition** Samples of each dressing were placed on agar plates inoculated with 0.2ml of a log-phase broth culture of each test organism. After incubation, the plates were examined for the presence of a zone of inhibition. If one was detected, the width was measured and the dressing was removed from the agar, placed on another agar plate and seeded as before with the same microorganism. This process was repeated a maximum of seven times or until no further zone of inhibition was produced during the previous test.

- **Challenge testing** 0.2ml of a log-phase culture of each microorganism was added to portions of each dressing measuring 40x40mm. The inoculated dressings were incubated for two hours, then transferred into 10ml of 0.1% peptone water (Oxoid) and vortexed to remove any remaining viable organisms. Serial dilutions were performed in triplicate on each extract, and the number of viable organisms present determined using a standard surface counting technique.

If viable organisms were recovered, the test was repeated as before using a four-hour contact period, and then again with a 24-hour contact period.

If no organisms were detected on a particular dressing after two hours, the dressing was placed in 10ml of tryptone soya broth (TSB) to detect very low levels of residual contamination.

As no inactivator for silver was used during this test, it is possible that any remaining low concentrations of silver ions present could have prevented the recovery of these organisms, potentially resulting in a false negative result.

- **Microbial transmission test** Here, a strip of dressing forms a bridge between two separate agar blocks in a Petri dish, one of which is sterile and the other inoculated with the test organism. This test determines the bacteria's ability to survive on the dressing surface and migrate along it from the contaminated agar to the sterile agar. A positive result suggests that it is possible that microorganisms could be transported laterally out of a contaminated wound onto the surrounding skin, or potentially move in the opposite direction from the intact skin into the wound itself.

Silver content

Samples of each dressing were sent to Sheffield Analytical Services to determine the total extractable silver content of each dressing by inductively coupled plasma optical emission spectroscopy (ICP-OES).

Results

Zone of inhibition test

The results of this method for the three test organisms are summarised in Table 1, which also includes results from the original paper.[1] There was considerable variation in ability to inhibit growth of the three test organisms. To further facilitate comparisons, a simple scoring system is also included. A dressing gets three points for each appearance in group A, two points for each appearance in group B and no points for each appearance in group C. The sum of these scores produces a very crude measure of the dressings' overall performance in this test.

Microbial challenge test

The results of this test are summarised according to the dressings' ability to produce a marked antimicrobial effect, arbitrarily defined as a 10^3 reduction in the number of viable organisms present at each time interval (Table 2). A similar scoring system to that outlined above has been devised to facilitate later comparisons. Due to the physical nature of the Arglaes material, it was not possible to include it in this series of tests.

Microbial transmission test

Results are summarised in Table 3. Again, both Tables 2 and 3 include results from the first paper.[1] Other than the control material, the only test samples to show any evidence of microbial transfer were Actisorb Silver 220 and Avance.

As previously discussed,[1] in the case of Actisorb Silver 220, microbiological migration occurred on only one sample, and probably took place across the nonwoven fabric outer sleeve of the dressing. No transfer occurred when only the inner core of the dressing was examined.

Evidence of transmission of bacteria was clearly visible on all three samples of Avance, as shown by prolific bacterial growth around the ends of the dressing on the surface of the sterile agar.

No transfer of *Candida albicans* took place on any of the sample tested including the control, which made the tests invalid.

Silver content of dressings

The total silver content of each dressing included in both papers is shown in Table 4 ranked by silver content, which indicates that major differences exist between the these products, with values ranging from 546 to 1.6mg/ $100cm^2$. Also included are the total scores achieved by each dressing in the various laboratory tests.

Discussion

The test methods were designed to compare the performance of the dressings under different simulated conditions of use.

The zone of inhibition method simulates the use of the products on moist or lightly exuding wounds and predicts the dressings' ability to kill or prevent bacterial growth in this situation.

In order to exert a significant antimicrobial effect in this test, a dressing must first absorb moisture from the agar to activate or release the silver held within its structure. This silver, in the form of silver ions, must then diffuse back down into the agar to exert its antimicrobial action.

The microbiological challenge test provides an indication of each dressing's ability to kill or prevent growth of predetermined numbers of bacteria applied directly in the form of a suspension, and thus to some extent reflects what may occur within dressings applied to more heavily exuding wounds.

The third test determines the bacteria's ability to survive and be transmitted along the dressing surface.

It was anticipated from the outset that the ability to exert a significant antimicrobial effect would be directly related to the total amount of silver present. The very crude scoring system described above seems to support this view. There is clearly a very strong association between the dressings' measured silver content and the scores they achieved in the laboratory tests, although two results for Silverlon and Contreet Ag require further comment.

Although it scored highly overall, the somewhat poor performance of Silverlon against *Staphylococcus aureus* in the challenge test was surprising given that it contained by far the highest concentration of silver of any of the dressings examined (four to five times the amount of the next two highest products). Similarly, although Contreet Ag performed well in some tests, it was disappointing in others.

While total silver content is important, other factors also influence a dressing's ability to kill microorganisms. These include the distribution of the silver within the dressing (whether it is present as a surface coating or is dispersed through the structure), its chemical and physical form (whether it is present in a metallic, bound or ionic state) and the dressing's affinity for moisture — a prerequisite for the release of active agents in an aqueous environment. Products in which the silver content is concentrated on the dressing surface rather than 'locked up' within its structure performed well, as did those in which silver was present in the ionic form.

Calgitrol, which contains a high concentration of silver, performed very well in all tests. This is probably because the silver, already in the ionic form, is concentrated on the dressing surface in a hydrophilic coating, which facilitates its rapid release.

Contreet Ag and Contreet-H, although containing broadly similar concentrations of silver, performed very differently in the first two tests. In the zone of inhibition test the hydrocolloid performed well, unlike Contreet Ag. In contrast, in the challenge test the foam-based Contreet Ag markedly outperformed the hydrocolloid.

The reasons for this are not entirely clear but may be related partly to differences in the fluid-handling characteristics of the two dressings. Under the test conditions, absorbency of Contreet Ag may be such that it created a suction gradient, continuously drawing fluid out of the agar and inhibiting the movement of solution containing silver ions in the reverse direction. Also, the foam had a tendency to curl away from the agar plate. In the challenge test, however, the organisms applied directly to the foam were destroyed by the silver ions released within its structure. This test probably more closely reflects the dressing's performance in the management of more heavily exuding wounds.

Aquacel contains ionic silver in a hydrophilic fibrous fleece. This material's fluid affinity is such that it was readily capable of drawing moisture out of the agar, which then released the silver ions. This enabled the dressing to exert significant antimicrobial activity on extended incubation, despite the relatively modest silver content.

Silvasorb, which also contains a relatively low concentration of ionic silver, showed broadly similar activity to Aquacel due to its hydrophilic structure.

Actisorb Silver 220, which contains low concentrations of metallic silver, showed evidence of only very limited antimicrobial activity.

Avance, which has the lowest silver content of the products examined, showed no evidence of any antimicrobial effect in any of the tests.

Considerable caution must be exercised when extrapolating the results of this or any laboratory-based study to the clinical situation as many factors determine a dressing's acceptability or clinical effectiveness, which may not become apparent in a laboratory model. For example, this study made no attempt to compare the fluid-handling properties of the various products or to determine their tissue compatibility or potential cytotoxic effects. All these issues were discussed at length.[1] Nevertheless, we believe our results may provide useful information to clinicians on one key aspect of the performance of this relatively new class of products.

This study was partly supported by Smith and Nephew

More detailed data on the results of the zone of inhibition and challenge tests are available from the author

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1 Thomas, S., McCubbin, P. A comparison of the antimicrobial effects of four silver-containing dressings on three organisms. J Wound Care 2003; 12: 3, 101-107.

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Table 1. Summary of zone-of-inhibition data

	S. aureus	*E. coli*	*C. albicans*
Group A (score 3)			
Products that show evidence of sustained activity over two or more days	Acticoat Aquacel Ag Calgitrol Ag Contreet-H Silverlon	Acticoat Calgitrol Ag Contreet-H Hydrocolloid Silverlon	
Group B (score 2)			
Products that produce a well-defined zone of inhibition at one time interval	Arglaes Power Silvasorb	Aquacel Ag Arglaes Powder	Acticoat Arglaes Powder Calgitrol Ag Contreet-H Silvasorb Silverlon
Group C (score 0)			
Products that produce no well-defined zone of inhibition in this test	Actisorb Silver 220 Avance Contreet Ag	Actisorb Silver 220 Avance Contreet Ag Silvasorb	Actisorb Silver 220 Aquacel Ag Avance Contreet Ag

Table 2. Summary of microbial challenge test results

	S. aureus	*E. coli*	*C. albicans*
Group A (score 4)			
Products that demonstrate marked antibacterial activity after two hours' incubation	Acticoat Calgitrol Ag	Acticoat Calgitrol Ag Contreet Ag Silverlon	Acticoat Calgitrol Ag Contreet Ag Silverlon
Group B (score 3)			
Products that demonstrate marked antimicrobial activity after four hours' incubation	Silverlon	Contreet-H Aquacel Ag Silvasorb	
Group C (score 2)			
Products that demonstrate marked antimicrobial activity after 24 hours' incubation		Actisorb Silver 220	
Group D (score 1)			
Products that demonstrate limited evidence of antimicrobial activity after 24 hours' incubation	Aquacel Ag Contreet-H Contreet Ag Silvasorb		Contreet-H Aquacel Ag Silvasorb
Group E (score 0)			
Products that demonstrate no convincing evidence of antimicrobial activity even on prolonged incubation	Actisorb Silver 220 Avance	Avance	Actisorb Silver 220 Avance

Table 3. Microbial transmission test

	S. aureus	*E. coli*	*C. albicans*
	Transfer ±	Transfer ±	Transfer ±
Acticoat	-	-	-
Actisorb Silver 220	+	+	-
Actisorb Silver 220*	-	-	-
Avance	+++	+++	-
Contreet-H	-	-	-
Control	+++	+++	-
Acticoat	-	-	-
Aquacel Ag	-	-	-
Calgitrol Ag	-	-	-
Contreet Ag	-	-	-
Silvasorb	-	-	-
Silverlon	-	-	-
Control	+++	+++	-

Each + indicates the results for a single test strip
*Only the inner core of Actisorb Silver 220 was used

Table 4. Silver content of the dressings

Product	Batch no.	Ag content ($mg/100cm^2$)	Total performance scores
Silverlon	102502-01	546	19
Calgitrol Ag	131-71	141	20
Acticoat	010814A-08 020214A	109 101	20
Contreet Ag	74853.01	47	9
Contreet-H	315768 267462 344046	31.2 32.4 31.4	13
Aquacel Ag	2H55863	8.3	10
Silvasorb	02082001	5.3	9
Actisorb Silver 220	0138-03 0135-04	2.9 2.4	2
Avance	01106947	1.6	0
Arglaes powder	527027	6.87mg/gram	

PROPERTIES OF ALGINATES

BY

R. H. McDOWELL

M.A., B.Sc., A.M.I.Chem.E.

ALGINATE INDUSTRIES LIMITED

WALTER HOUSE · BEDFORD STREET · STRAND · LONDON · W.C.2

Exhibit K

that guluronic acid residues as well as those of mannuronic acid were present. This has since been confirmed by other workers[11, 12]. It is not quite certain whether polymannuronic acid and polyguluronic acid chains exist independently in alginic acid, or whether the two uronic acids occur in the same chains. It has been found that alginates can be fractionated by precipitation with manganese[13] salts and with potassium chloride[14], and that the two types of polyuronide are concentrated in the different fractions[15], but on the other hand, di- and triuronides apparently containing both mannuronic and guluronic acid have been isolated from the hydrolysis products of alginic acid[16].

Manufacturers and users of alginates have long been aware of quantitative differences in the behaviour of alginates from different species of algae: it is now known that the proportions of mannuronic and guluronic acid vary from one species to another and this may go a long way towards accounting for these differences. However, with the present state of our knowledge the different alginates must still be referred to by their origin rather than by proportions of uronic acids.

It must be emphasised that the physical and chemical properties of alginates from different sources are the same from a qualitative point of view, and the differences are to be found only when precipitation reactions and the physical properties of precipitated or mixed salt alginates are examined quantitatively. This is to be expected from the similarity of structures of the two polyuronides which they contain in differing proportions.

A study of sodium alginates from different sources was made by Vincent, Goring and Young[17]. Properties investigated included ionic mobilities and sedimentation constants, and the samples covered a wide range of molecular weight as shown by the viscosities of their solutions. No significant variation in the properties examined could be related to the origin of the products. It is therefore reasonable to continue to use the name alginic acid for the products from different species, but published work giving quantitative results without reference to seaweed origin should be treated with caution. The degree of polymerisation, which can be varied by adjusting the methods of extracting the alginates from the different algae, can also modify other properties quantitatively.

when the carefully purified acid has been dried for 24 hours in vacuo over P_2O_5. But normal oven drying of the salts of alginic acid obtained from different species of algae have given figures for the equivalent of the free acid consistently close to 194, and this is the most satisfactory figure to use in calculations. The discrepancy between the actual and theoretical figures is most easily accounted for by the close assocation of one molecule of water with each uronic acid unit, but this has not definitely been proved. It should be noted that Astbury[9], obtained sharp X-ray diagrams from alginic acid fibres only when they contained some moisture.

Molecular Weight

When present in living algae, alginic acid can be in a very highly polymerised state, as is shown by the extremely viscous solutions of the sodium salt obtained by carefully controlled extraction.

By differences in method of preparation, alginates with a wide range of molecular weights can be obtained. Molecular weights have been measured osmotically (Donnan and Rose[19]) and by the ultracentrifuge method (Saverborn[20], Cook and Smith[21]).

The different results obtained are critically reviewed in the paper by Cook and Smith[21] and while there are considerable uncertainties in the calculations, there is little doubt that commercial high viscosity alginates have molecular weights of well over 150,000, corresponding to a degree of polymerisation (D.P.) of about 750. Further work on closely fractionated samples would be valuable as all those examined have contained rather a wide range of molecular size. The relation between molecular weight and viscosity of solutions is considered on page 19 under " Viscosity."

Stability of Alginates

Highly polymerised alginic acid depolymerises to give alginic acid of lower molecular weight, but the lower polymers thus formed are very stable at normal temperatures. Alginic acid with a D.P. of about 40 will remain unchanged for years at 10° to 20°C.

It is difficult to bring about complete breakdown to the uronic acids: heating with an acid is necessary, and in these conditions

there is some breakdown of the uronic acid, the guluronic acid decomposing more rapidly than the mannuronic acid. Methods for isolating mannuronic acid from the hydrolysis products have been published[22, 23].

The salts of alginic acid are much more stable, and sodium alginate of D.P. about 500 has been stored at 10° to 20°C. with no observable change in three years. Degradation can be observed with the more highly polymerised alginates, and can become serious if the product is stored at higher temperatures (say above 50°C.). The rate of depolymerisation is greater in the presence of moisture than in the dry state and increases as the proportion of moisture is increased.

At much higher temperatures there is actual breakdown of the uronic acid units both with the free acid and its salts, and at temperatures not much above 200°C. there is rapid evolution of one molecule of CO_2 for every uronic acid unit. This has been developed as a method of determining alginates by Perlin[24].

Similar considerations apply to solutions of alginates. A neutral solution of sodium alginate of a moderate degree of polymerisation, suitably protected from microbiological attack, can be kept for years in a temperate climate with very little change in viscosity. Solutions of highly polymerised alginates decrease in viscosity at ordinary room temperatures and solutions of all alginates do so at higher temperatures. The presence of acid or alkalis which bring the pH to below 5 or above 9 will accelerate depolymerisation.

Dissociation Constant of Alginic Acid

In water solutions of partially neutralised alginic acid, the titration curve is different from that of a monobasic acid, the dissociation " constant " getting progressively lower as the acid is neutralised, and in all cases being lower than that of monomeric mannuronic acid; this is due to the negative charge on the alginate ion. In salt solutions, however, the effect of the charge is suppressed and Saric and Schofield[25], working with N potassium chloride found that alginic acid had a pK of 2.95 over the range pH 2.8 to pH 6. Below pH 2.8 the monobasic acid curve was not followed exactly, but this is not surprising as the alginic acid is precipitated below pH 3.4

Alginate Solutions

General Discussion

The soluble alginates have properties typical of hydrophilic colloids. For example; their solutions are much more viscous than those of simple substances at the same concentration; alginates can be separated from substances of low molecular weight by dialysis; and the process of drying out a solution is readily reversible. (As the alginate is molecularly dispersed, it is reasonable to refer to a solution of the alginate in water). It is now usual to refer to alginates and similar substances as polyelectrolytes. They have large molecules and in solution carry an electric charge by reason of their electrolytic dissociation.

It is generally found that an alginate is either practically insoluble or is completely miscible with water, unlike substances of low molecular weight where the solid will exist in equilibrium with a saturated solution containing a considerable amount of the dissolved solid. This type of behaviour is usual with high polymers, but it should be noted that in some cases there can be separations of fractions of different degrees of polymerisation, the more highly polymerised material remaining undissolved in a solution of the lower polymers. It has also been found that there can be some additional separation into fractions having higher or lower ratios of mannuronic to guluronic residues.

In many cases an alginate can quite definitely be described as insoluble, as no trace of dissolved material can be detected when the alginate is mixed with water. This is the case with most metallic alginates. Others, such as sodium alginate, are definitely very soluble in water, but in dealing with mixed salts, or with limiting concentration of precipitants it is not possible to give the exact limiting conditions for solubility. Difficulties in the way of quantitative examination are:

(*a*) The high viscosity of even moderately concentrated alginate solutions, which makes the separation of two phases difficult.

(*b*) The highly swollen state of precipitated alginates.

(*c*) The extremely slow attainment of equilibrium between phases.

Figures for limits are therefore only approximate. It is sometimes difficult to be certain whether an alginate is completely dissolved or not. For example, mixtures of sodium alginate and water in all proportions ranging from water through viscous solutions and pastes to hard solids can be made. Whether these are all one phase systems, or whether in some two phases are present, is uncertain.

Alginates are essentially hydrophilic and the simple alginates are insoluble in common non-aqueous solvents, although sodium alginate can be dissolved, for example, in molten urea. The addition of water miscible liquids, such as alcohol, to aqueous solutions of most alginates will cause precipitation. The amount of liquid which can be added without causing precipitation depends on the base combined with alginic acid. While sodium alginate is precipitated by the addition of 20% to 30% of alcohol, amine salts, particularly those of the higher amines are soluble in mixtures of alcohol and water containing a high proportion of alcohol. There is now available an amine alginate (Collatex P) which is soluble in lower alcohols but insoluble in water. Ammonium alginate requires more alcohol for precipitation than sodium alginate, but less than that of most of the salts of organic bases. The exact amount required depends in all cases on the concentration of the solution and the degree of polymerisation of the alginate.

In the following discussion of solubility relations, it is the solubility in water that is meant in each case.

Solubilities of Individual Alginates

Alginic acid is insoluble. Considering its chemical nature this is at first sight surprising, and here a comparison with cellulose and pectin is illuminating (see Fig. 1, page 4). The X-ray examination of both alginic acid and cellulose has shown them to be largely in a crystalline state, so that they have very regular structures, affording ample opportunity for hydrogen bonding between polymer chains at regular intervals. On the other hand pectin is the partial methyl ester of pectic acid, the carboxyl groups being esterified at random. It is therefore understandable that the energy required to separate the molecular chains, the first step towards solution, is much

greater for alginic acid and cellulose than for pectin. Pectin is in fact completely miscible with water, but removal of the methyl group by hydrolysis gives pectic acid, insoluble in water, and similar in properties to alginic acid. In the same way some derivatives of cellulose and of alginic acid, in which irregularities in the chain reduce the opportunity for crystallisation, are soluble in water. Examples are the partial methyl ether of cellulose and the partial propylene glycol ester of alginic acid.

The presence of the acid groups in alginic acid affords a very easy method of bringing it into solution, as by the formation of a highly ionised salt, electrostatic forces can shift the free energy balance in favour of solution. In titrating alginic acid with sodium hydroxide, about three-quarters of the carboxyl groups have to be neutralised to bring about solution. This takes place at about pH 3.5. The transition is quite sharp. For example Saric and Schofield[25] found that at pH 3.6 all their alginate was dissolved, but at pH 3.3 the whole could be centrifuged out as alginic acid.

The salts of the alkali metals, ammonia, and many organic bases are soluble in water while those of most of the di- and polyvalent metals are insoluble. The insolubility of these alginates is probably due to a combination of forces, but it is understandable that, even if completely ionised, divalent cations could keep the alginate ions sufficiently closely associated to prevent solution. The valency of the base is not the sole deciding factor as magnesium alginate is soluble, while silver alginate is insoluble.

The solubilities of some of the common alginates and their colours are given in Table 1. In the last column some procedures for obtaining clear solutions, free from precipitated salts, are included. These methods involve chemical transformation of the alginate, but it is sometimes useful to prepare solutions in this way rather than starting with a soluble alginate. Solution brought about by adding ammonium hydroxide is readily reversed by removal of the ammonia so that insoluble films can be formed by evaporation. Insoluble alginates can also be brought into solution by reaction with the alkali salt of an anion which forms an insoluble

salt with the metal combined with the alginate, but in this case the resulting solution contains the insoluble salt as a suspension or sediment.

TABLE I

Alginate Salt	Colour	Solubility in Water	Dissolved by Adding
Sodium, potassium ammonium, magnesium	White	Soluble	
Triethanolamine	Pale Yellow	Soluble	
Calcium	White	Insoluble	Sodium metaphosphate
Aluminium	White	Insoluble	Ammonium hydroxide
Zinc	White	Insoluble	Ammonium hydroxide and ammonium salt
Copper	Blue	Insoluble	Ammonium hydroxide
Chromium	Grey-green	Insoluble	
*Iron (ferrous)	Pale-green	Insoluble	
Iron (ferric)	Brown	Insoluble	
Silver	White (darkens in light)	Insoluble	Ammonium hydroxide

* There has been some doubt about the solubility of ferrous alginate—for further discussion see Page 29.

Dissolving Soluble Alginates

Soluble alginates are easily and quickly dissolved in water with the aid of a high speed stirrer. Other methods can be used but are not so rapid. Details are given in the A.I.L. leaflets " Dissolving Alginates " and " Dissolving Readily Soluble Alginates."

It is advisable to use soft water when preparing alginate solutions. This is essential when dilute solutions are being made; the higher the ratio of water to alginate, the more serious would be small amounts of dissolved calcium in the water. The extreme case of dilution arises when an alginate is being washed out of, for example, a fabric, and it is useful then to include sodium metaphosphate or carbonate in at least one lot of the wash water.

TABLE II

Compatibilities of Alginates in Solution

A. COMPATIBLE

Some common substances, high proportions of which can be included in aqueous alginate solutions without causing precipitation.

Polyhydric alcohols	Ethylene glycol, glycerol, sorbitol, mannitol.
Carbohydrates	Simple sugars, starch, soluble cellulose derivatives.
Gums	Acacia, tragacanth, locust bean, guar, karaya, carrageenin, pectin.
Salts	Most salts of the alkalis, ammonium and magnesium, e.g. sodium sulphate, sodium carbonate, borax, magnesium sulphate.
Dyestuffs	Directs, vats, solubilised vats, Rapidogens, many acid dyes, pigment dyestuffs and binders.
Proteins	Gelatine, egg albumen, casein, vegetable proteins (calcium assumed absent or sequestered).
Miscellaneous	Urea, phenol, emulsified oils and resins, boric acid.

B. INCOMPATIBLE

Some substances which even in low concentration precipitate alginates from solution.

Salts	Soluble ionised salts of alkaline earths and heavy metals.
Acids	Strong acids (sufficient to bring the pH to below 3.5 in alginate salt solutions, 2.5 in propylene glycol solutions.
Organic substances	Cationic detergents, positively charged colloids.

Precipitation of Alginates without Chemical Change

Although precipitation is generally due to the formation of a water insoluble alginate, some substances can bring about precipitation without chemical change; the action of water miscible non solvents for alginates has already been mentioned (page 10). Precipitation can also be brought about by moderate or high concentrations of some simple electrolytes. This is presumably a salting out effect similar to that which takes place with many other colloids, but quantitatively the effect of different salts is difficult to understand. For example, certain types of sodium alginate are precipitated by N (5.8 gm./100 ml.) sodium chloride, but will dissolve in 3 N (21.3 gm./100 ml.) sodium sulphate.

Kelco
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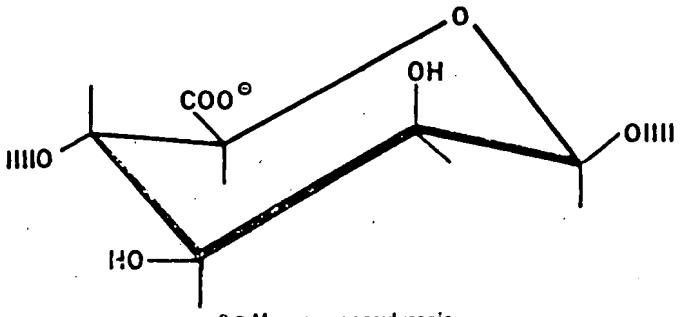
Kelco Algin/hydrophilic derivatives of alginic acid for scientific water control

Second Edition

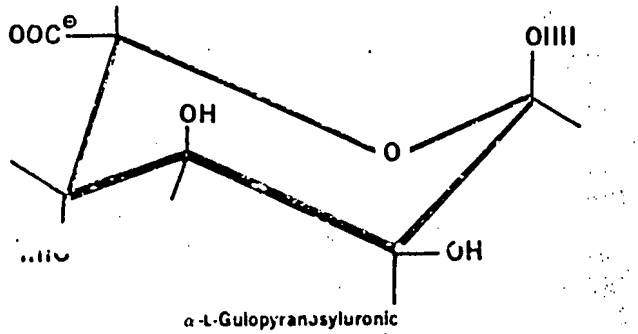
Exhibit L

Figure 4-A. Conformation of mannuronic acid.



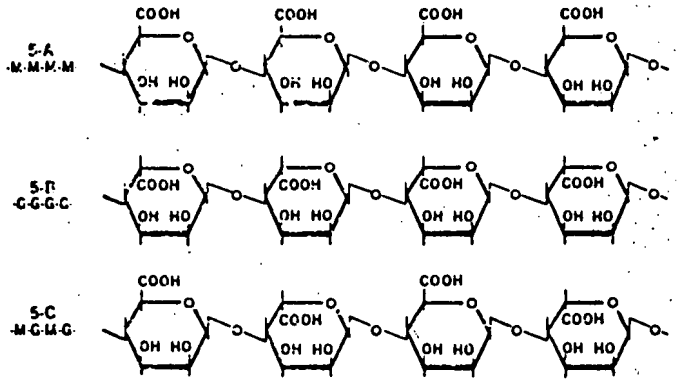
β-D-Mannopyranosyluronic acid unit—C1 conformation

Figure 4-B. Conformation of guluronic acid.



α-L-Gulopyranosyluronic acid unit—1C conformation

Figure 5-A-C. Structure of the polymer segments contained in alginic acid.



STRUCTURE

Algin, the polysaccharide extracted from the brown seaweeds, *Phaeophyceae*, has only recently been chemically identified.

Although purified alginic acid was prepared by Krefting in 1896, the first report on its structure was made in 1930 by Nelson and Cretcher, who claimed it was a D-mannuronic acid polymer. Later investigation by Hirst and co-workers determined alginic acid to be composed of D-mannuronic acid connected by β1→4 linkages *(Hirst et al. 1939)*. In 1955, Fischer and Dörfel, using paper chromatography, determined that L-guluronic acid was also present as a major component of alginic acid. Vincent *(1960)* and Hirst, Percival, and Wold *(1964)* showed that at least some of the alginic acid molecules contained both mannuronic acid (Figure 4-A) and guluronic acid (Figure 4-B) by use of partial acid hydrolysis to isolate oligomers containing both uronic acids.

Improvements in the techniques for the hydrolysis, separation, and analysis of alginic acid have allowed accurate determinations of the composition of alginic acid from different sources to be made. Table 2 shows the composition of alginic acid obtained from commercially important brown algae.

The presence of three kinds of polymer segments in alginic acid from various brown algae has been shown by mild acid hydrolysis *(Haug et al. 1966, 1967a, and 1967b)*. One segment consists essentially of D-mannuronic acid units (Figure 5-A); a second segment consists essentially of L-guluronic acid units (Figure 5-B); and the third segment consists of alternating D-mannuronic acid and L-guluronic acid residues. (Figure 5-C).

The proportions of the three polymer segments in alginic acid samples from different sources have been determined by Haug and co-workers *(1966 and 1967a)* using partial acid hydrolysis to separate the alginic acid into homopolymeric and alternating segments. Penman and Sanderson *(1972)* have determined the proportion of polymannuronic acid and polyguluronic acid segments by p.m.r. spectroscopy. Table 3

Table 2

Mannuronic Acid (M) and Guluronic Acid (G) Composition of Alginic Acid Obtained from Commercial Brown Algae

Species	Mannuronic Acid Content (%)	Guluronic Acid Content (%)	M/G Ratio	M/G Ratio Range
Macrocystis pyrifera	61	39	1.56[a]	–
Ascophyllum nodosum	65	35	1.85 (1.1)[a]	1.40-1.95[b]
Laminaria digitata	59	41	1.45[a]	1.40-1.60[b]
Laminaria hyperborea (stipes)	31	69	0.45[a]	0.40-1.00[b]
Ecklonia cava and Eisenia bicyclis	62	38	1.60[a]	–

(a) Data of Haug *(1964)* and Haug and Larsen *(1962)* for commercial algin samples. Of the two ratios shown for *Ascophyllum nodosum*, the algin sample manufactured in Canada has the higher M/G value; the lower ratio corresponds to a European sample.

(b) Data of Haug *(1964)* showing the range in composition for mature algae collected at different times at each of several locations.

Table 3
Proportions of Polymannuronic Acid, Polyguluronic Acid, and Alternating Segments in Alginic Acid Isolated from Brown Algae[a]

Source	Polymannuronic Acid Segment (%)	Polyguluronic Acid Segment (%)	Alternating Segment (%)
Macrocystis pyrifera	40.6	17.7	41.7
Ascophyllum nodosum	38.4	20.7	41.0
Laminaria hyperborea	12.7	60.5	26.8

(a) Data of Penman and Sanderson *(1972)*

shows the proportions of polymannuronic acid, polyguluronic acid, and alternating segments in three commercial alginic acid samples.

The differences in composition and fine structure indicated in Tables 2 and 3 account for the differences in properties and functionality of alginates isolated from different species of brown algae.

The theoretical equivalent weight of alginic acid is 176, but the bound water within the molecule results in measured values close to 194 *(Haug 1964)*.

The dissociation constant for alginic acid depends on the ratio of mannuronic acid to guluronic acid. Haug *(1964)* has reported the following dissociation constants:

Acid	pKa
Mannuronic Acid	3.38
Guluronic Acid	3.65

Recently it has been shown using p.m.r. spectroscopy and model building with a computer, that alginic acid salts, such as sodium alginate, in aqueous solution are highly hydrated polyelectrolytes in the extended ribbon conformation.

X-ray diffraction studies on fibers of alginic acid and polarized infrared spectroscopy of oriented films have resulted in information on the crystalline structure of polymannuronic acid and polyguluronic acid.

The shape of the polymannuronic acid chain is similar to that found in other $\beta 1 \rightarrow 4$ linked hexosans such as cellulose. The mannuronic acid is in the C1 conformation and, therefore, di-equatorially linked (Figure 6-A). Polymannuronic acid is a flat, ribbon-like molecule (Figure 7-A), the conformation of which appears to be stabilized by the formation of an intra-molecular hydrogen bond between the hydroxyl group on carbon 3 of one unit and the ring oxygen atom (O_5) of the next sugar unit in the chain *(Atkins et al. 1971)*. The chains themselves are bonded into sheets by means of hydrogen bonds formed between the hydroxyl of the carboxyl group and the oxygen atom on carbon 3 in sugar units in parallel chains and between the axial hydroxyl group on carbon 2 and the oxygen atom of the carboxyl group in antiparallel chains.

Figure 6-A. Repeating unit of polymannuronic acid.

The glycosidic linkage is 1e→4e, i.e., di-equitorial.

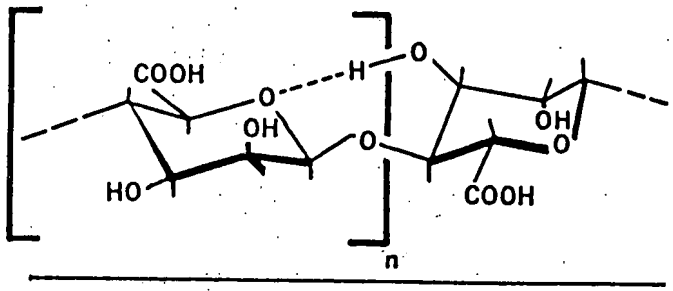
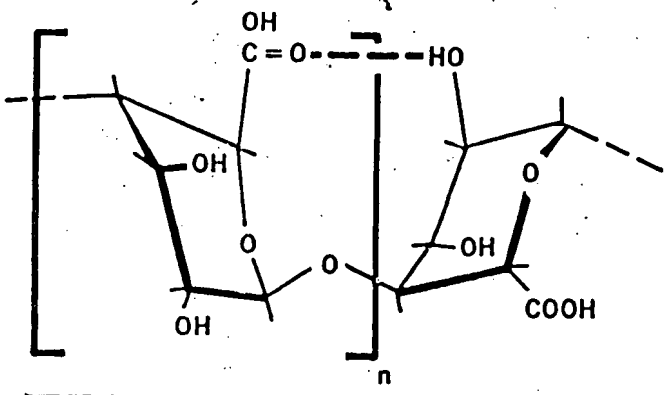


Figure 6-B. Repeating unit of polyguluronic acid showing the difference in conformation.

The glycosidic linkage is 1a→4a, i.e., di-axial.



The shape of the polyguluronic acid chain is quite different from that of polymannuronic acid. Polyguluronic acid is a buckled, ribbon-like molecule (Figure 7-B) in which the guluronic acid is in the 1C conformation and, therefore, di-axially linked (Figure 6-B). The buckled, ribbon-like conformation is stabilized by an intramolecular hydrogen bond between the hydroxyl group on carbon 2 and the oxygen atom of the carboxyl group in adjacent units. The interchain bonds are more complicated than in the case of polymannuronic acid and involve water molecules. A water molecule is in such a position that it functions twice as a hydrogen bond donor and twice as an acceptor, the hydrogen bonds so formed being in the range of 2.7Å-2.9Å. In accord with den-

IV Toxicity, Regulatory, and Environmental Information

The toxicological properties of alginates have been extensively investigated and summarized *(McNeely and Kovacs 1975; Anon. 1972)*.

One of the earliest studies investigated the digestibility of sodium alginate in male albino rats during a 10-day period. The results indicated that the digestibility of sodium alginate ranged from 3 to 88% depending upon the level fed *(Nilson and Lemon 1942; Nilson and Wagner 1951)*. Sodium alginate and propylene glycol alginate were incorporated in the diets of rats, mice, chicks, cats, and guinea pigs for prolonged periods without any deleterious effects.

In a series of separate experiments, Morgan *(1959)* placed 5% sodium alginate, standardized sodium alginate (DARILOID), and propylene glycol alginate in the diet of 40 male and 40 female albino rats for two years. Neither the parent generation (the F_1 generation) nor their progeny (the F_2 generation) showed any adverse effects from this prolonged exposure to alginates in their diet *(Johnston et al. 1964)*.

An investigation by Woodard *(1959)* indicated that incorporating 5 to 15% sodium alginate or propylene glycol alginate in the diet of purebred beagle dogs for the period of one year caused no harmful effects.

Since no satisfactory data were reported in the literature on the acute oral toxicity (LD-50) of alginates, Kelco recently commissioned the Woodard Research Corporation to conduct studies on two algin compounds. The results indicated that the administration of 5 g/kg body weight to rats within a 24-hour period caused no mortalities nor signs of toxicity *(Knott 1972)*.

A recent study indicated that the subcutaneous injection of mice with alginic acid caused no carcinogenic activity *(Epstein et al. 1970)*. An investigation of the effect of degraded and undegraded sodium alginate on the colon of guinea pigs indicated that alginate did not cause ulcerative colitis either in its degraded or undegraded form *(Watt and Marcus 1971)*. The investigation of the metabolic pathway of propylene glycol alginate indicated that the alginate moiety of the molecule remains unabsorbed, while the non-toxic propylene glycol produced by the hydrolysis of the propylene glycol alginate is absorbed through known metabolic pathways *(Sharratt and Dearn 1972)*. Sodium alginate and propylene glycol alginate have also been shown by recent investigations to possess no eye or skin irritation properties *(Johnston 1972a and 1972b)*.

In summary, numerous studies have attested to the high level of safety of alginates in food use.

Government Regulations and Labeling Requirements

Ammonium alginate, calcium alginate, potassium alginate, and sodium alginate are included in a list of stabilizers that are generally recognized as safe (GRAS) under 21 CFR 121.101. Propylene glycol alginate is approved as a food additive under 21 CFR 121.1015 for use as an emulsifier, stabilizer, or thickener in foods in accordance with good manufacturing practice.

The use of the edible salts of alginic acid, as well as propylene glycol alginate, is approved in all appropriate standard of identification regulations. In these standards, alginates are either specifically mentioned by their common and usual name or are included under the provision of "safe and suitable optional ingredients." For maximum allowable usage levels, each regulation must be consulted separately.

Propylene glycol alginate is approved for use in defoaming agents (21 CFR 121.1099), in coating on fresh citrus fruit (21 CFR 121.1179), as an inert pesticide adjuvant (40 CFR 180.1001), and as a component of paper and paperboard in contact with aqueous and fatty foods (21 CFR 121.2526).

Ammonium alginate and sodium alginate are approved for use as boiler water additives under 21 CFR 121.1088. The edible salts of alginic acid are also approved for use as a component of paper and paperboard in contact with aqueous and fatty foods under 21 CFR 121.2526, specifying that substances generally recognized as safe (GRAS) can be used.

KELGIN W and KELCOSOL, both of which are sodium alginates, are approved by the United States Public Health Service as coagulant aids for water treatment at maximum concentrations of 2 mg/l *(Anon. 1970)*.

The Food Chemicals Codex contains monographs on alginic acid, ammonium alginate, calcium alginate, potassium alginate, sodium alginate, and propylene glycol alginate *(National Research Council 1972)*. Monographs on sodium alginate and alginic acid are also included in the National Formulary *(Anon. 1975)*.

Alginic acid and its edible salts, as well as propylene glycol alginate, are included in the approved emulsifier/stabilizer lists published by the EEC and the Council of Europe. The FAO/WHO Joint Expert Committee established an Average Daily Intake (ADI) limit of 50 mg/kg for alginic acid and its edible salts and 25 mg/kg for propylene glycol alginate.

Biological Oxygen Demand

Biological and chemical oxygen demand data for KELGIN MV (sodium alginate), SUPERLOID (ammonium alginate), and KELCOLOID O (propylene glycol alginate) have been determined. These data are tabulated below:

Table 4
Biological Oxygen Demand[1]

Product	5-Day	10-Day	20-Day	C.O.D.
KELGIN MV	285	394	408	665
SUPERLOID	334	469	499	720
KELCOLOID O	124	184	266	1,238

(1) Data reported as mg Oxygen/g Substrate

V Kelco Alginates

The alginic acid derivatives produced by Kelco vary in a great many ways. Available for use by processors are the sodium, potassium, ammonium, and calcium salts of alginic acid as well as the acid itself. Mixed salts such as sodium-calcium and ammonium-calcium are also produced. Kelco also manufactures propylene glycol ester of alginic acid, the only organic derivative made commercially.

The normal variables include:

1. Molecular weight (viscosity grade)
2. Calcium content
3. Particle form—granular, fibrous
4. Mesh size of the particles or fibers.

On the following pages are basic data for several types of Kelco alginates (Table 5) and a tabulation of physical properties of many of these products (Table 6 A-J). The viscosity/concentration relationships for several of the water-soluble alginates are shown in Figures 10 A-F. Table 6-K illustrates the typical nutritional values of Kelco alginates.

Table 5
Typical Physical Properties

	Alginic Acid	Refined Sodium Alginate	Specially Clarified Sodium Alginate	Ammonium Alginate	Propylene Glycol Alginate
Moisture Content	7%	13%	9%	13%	13% max.
Ash	2%	23%	23%	2%	10% max.
Color	White	Ivory	Cream	Tan	Cream
Specific Gravity	—	1.59	1.64	1.73	1.46
Bulk Density (lbs/cu ft)	—	54.62	43.38	56.62	33.71
Browning Temp., °C	160	150	130	140	155
Charring Temp., °C	250	340, 460	410	200	220
Ashing Temp., °C	450	480	570	320, 470	400
Ignition Temp., °C	*	*	*	*	*
Heat of Combustion (Cal/g)	2.80	2.50	2.44	3.04	4.44
As a 1% Solution (Dist. water)					
Heat of Solution (Cal/g Soln.)	0.090	0.080	0.115	0.045	0.090
Refractive Index (20°C)	—	1.3343	1.3342	1.3347	1.3343
pH	2.9	7.5	7.2	5.5	4.3
Surface Tension (Dynes/cm)	53	62	70	62	58
Freezing Point Depression, °C	0.010	0.035	0.020	0.060	0.030

*Spontaneous combustion did not occur in an air environment.

Table 6-A
Refined Sodium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELGIN® HV	Ivory	Granular	30	800	10,000	Neutral
KELGIN MV	Ivory	Granular	30	400	6,000	Neutral
KELGIN F	Ivory	Granular	80	300	4,000	Neutral
KELGIN LV	Ivory	Granular	40	60	500	Neutral
KELGIN XL	Ivory	Granular	40	30	160	Neutral
KELGIN RL	Ivory	Granular	40	10	30	Neutral
KELVIS®	Ivory	Granular	150	760	9,000	Neutral

Table 6-B
Industrial Sodium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELTEX®	Tan	Granular	20	800	10,000	Neutral
KELTEX P	Tan	Granular	80	765	9,000	Neutral
KELTEX S	Tan	Granular	20	1,300	—	Neutral

Table 6-C
Specially Clarified Low-Calcium Sodium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELCO-GEL® HV	Cream	Fibrous	80	400	3,500	Neutral
KELCO-GEL LV	Cream	Fibrous	150	50	250	Neutral
KELCOSOL®	Cream	Fibrous	80	1,300	15,000	Neutral
KELCO-PAC	Ivory	Granular	20	55	280	Neutral
KELTONE®	Cream	Fibrous	150	400	3,500	Neutral

Table 6-D
Refined Ammonium Alginate

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
SUPERLOID®	Tan	Granular	20	1,500	17,000	5.5

*These data were obtained using a Brookfield Model LVF Viscometer at 60 rpm with the appropriate spindle.

Table 6-E
Refined Potassium Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELMAR®	Cream	Granular	100	270	3,200	Neutral
Improved KELMAR	Cream	Fibrous	80	400	4,500	Neutral

Table 6-F
Propylene Glycol Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELCOLOID® HVF	Cream	Fibrous	80	400	7,000	4.0
KELCOLOID DH	Cream	Agglomerated[1]	20	400	7,000	4.0
KELCOLOID D	Cream	Fibrous	40	170	2,000	4.4
KELCOLOID LVF	Cream	Fibrous	80	120	1,200	4.0
KELCOLOID O	Cream	Fibrous	80	25	130	4.3
KELCOLOID DO	Cream	Agglomerated[1]	20	25	130	4.3
KELCOLOID S	Cream	Fibrous	80	20	115	4.0
KELCOLOID DSF	Cream	Agglomerated[1]	60	20	115	4.0

[1]*Agglomerated for improved dispersion.*

Table 6-G
Sodium Alginates Treated for Improved Dispersion

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELGIN QH	Ivory	Granular	30	400	6,000	Neutral
KELGIN QM	Ivory	Granular	30	180	1,800	Neutral
KELGIN QL	Ivory	Granular	30	30	160	Neutral

Table 6-H
Miscellaneous Alginates

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELSET®[1]	Light Ivory	Fibrous	80	—	Soft Gel	Neutral
KELTOSE®	Ivory	Granular	80	—	Soft Gel	Neutral
MARGEL®	Cream	Granular	100	—	250	9.4

[1]self-gelling

*These data were obtained using a Brookfield Model LVF Viscometer at 60 rpm with the appropriate spindle.

Table 6-I
Alginic Acid

Product	Color	Form	Approx. Mesh	Viscosity* 1%	Viscosity* 2%	pH
KELACID®	White	Fibrous	80	Insoluble		3

Table 6-J
Dairy Stabilizers and Stabilizer/Emulsifiers (Milk Soluble)

Product	Color	Form	Approx. Mesh	pH
DARILOID®	Light Ivory	Granular	40	10.0
Concentrated DARILOID	Light Ivory	Granular	40	10.0
DARILOID K	Light Ivory	Granular	14	4.5
DARILOID KB	Light Ivory	Granular	14	5.4
DARILOID Q	Light Ivory	Granular	100	10.2
DARILOID QH	Light Ivory	Granular	100	10.2
DARILOID XL	Light Ivory	Granular	40	10.0
Concentrated DARILOID XL	Light Ivory	Granular	40	10.0
DRICOID® KB	Light Ivory	Granular	14	5.4

These data were obtained using a Brookfield Model LVF Viscometer at 60 rpm with the appropriate spindle.

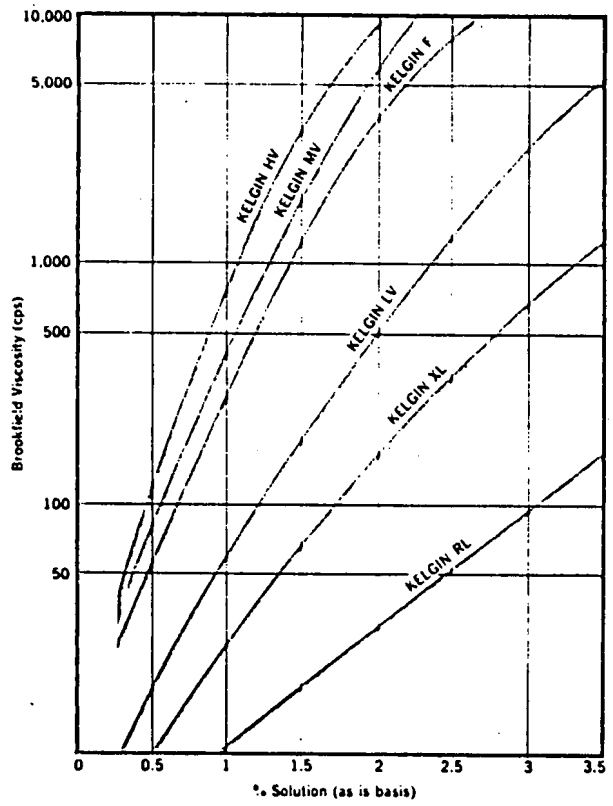
Table 6-K
Typical Nutritional Data of Kelco Alginates

Product	Cal.*	Carbo-hydrates (%)	Protein	Fat	Vitamins	Minerals (%) Na	Ca	Mg	K	P
COCOLOID®	2.2	77	Nil	Nil	Nil	7.4	0.6	0.03	0.30	1.1
Concentrated DARILOID	1.1	69	Nil	Nil	Nil	13.3	0.7	0.01	0.03	2.6
Concentrated DARILOID XL	1.1	69	Nil	Nil	Nil	13.3	0.7	0.01	0.03	2.6
Concentrated DARILOID KB	2.8	65	Nil	Nil	Nil	1.1	0.7	0.05	0.20	Nil
DARILOID	1.6	71	Nil	Nil	Nil	11.8	0.5	0.01	0.03	2.1
DARILOID K	2.4	76	Nil	Nil	Nil	1.7	0.6	0.01	0.30	Nil
DARILOID KB	3.3	86	Nil	Nil	Nil	0.5	0.2	0.01	0.10	Nil
DARILOID Q	2.2	77	Nil	Nil	Nil	7.3	0.4	0.01	0.01	1.9
DARILOID QH	2.2	77	Nil	Nil	Nil	7.3	0.5	0.01	0.02	1.9
DARILOID XL	1.5	71	Nil	Nil	Nil	11.1	0.5	0.01	0.02	1.9
DRICOID	3.8	47	Nil	26%	Nil	6.9	0.3	0.01	0.02	1.1
DRICOID KB	4.6	60	Nil	23%	Nil	0.5	0.2	0.01	0.10	Nil
SHERBELIZER	3.3	87	Nil	Nil	Nil	0.6	0.4	0.08	0.50	Nil
KELCOLOID D	2.4	70	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID DH	2.4	72	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID DO	2.5	70	Nil	Nil	Nil	1.2	0.3	0.01	0.02	0.1
KELCOLOID DSF	2.5	70	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID HVF	2.4	72	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID LVF	2.4	72	Nil	Nil	Nil	1.4	1.0	0.01	0.02	Nil
KELCOLOID O	2.5	69	Nil	Nil	Nil	1.2	0.3	0.01	0.02	0.1
KELCOLOID S	2.5	69	Nil	Nil	Nil	1.2	0.3	0.01	0.02	0.1
KELTOSE	1.3	81	Nil	Nil	Nil	0.4	3.7	0.10	0.02	Nil
Improved KELMAR	1.2	76	Nil	Nil	Nil	0.4	0.3	0.01	9.40	Nil
KELACID	1.5	92	Nil	Nil	Nil	1.2	1.2	0.01	0.02	Nil
KELCO-PAC	1.3	79	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELCO-GEL HV	1.3	80	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELCO-GEL LV	1.3	80	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELCOSOL	1.3	80	Nil	Nil	Nil	7.7	1.0	0.01	0.01	Nil
KELMAR	1.2	75	Nil	Nil	Nil	0.4	1.2	0.01	9.40	Nil
KELSET	1.3	81	Nil	Nil	Nil	6.5	3.0	0.02	0.10	Nil
KELTONE	1.3	81	Nil	Nil	Nil	9.4	0.2	0.01	0.10	Nil
KELVIS	1.3	80	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
MARGEL	1.0	64	Nil	Nil	Nil	5.5	8.7	0.01	0.03	Nil
KELGIN F	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN HV	1.3	80	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN LV	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN MV	1.3	80	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN RL	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil
KELGIN XL	1.3	79	Nil	Nil	Nil	8.4	1.2	0.02	0.01	Nil

*kilocalories per gram

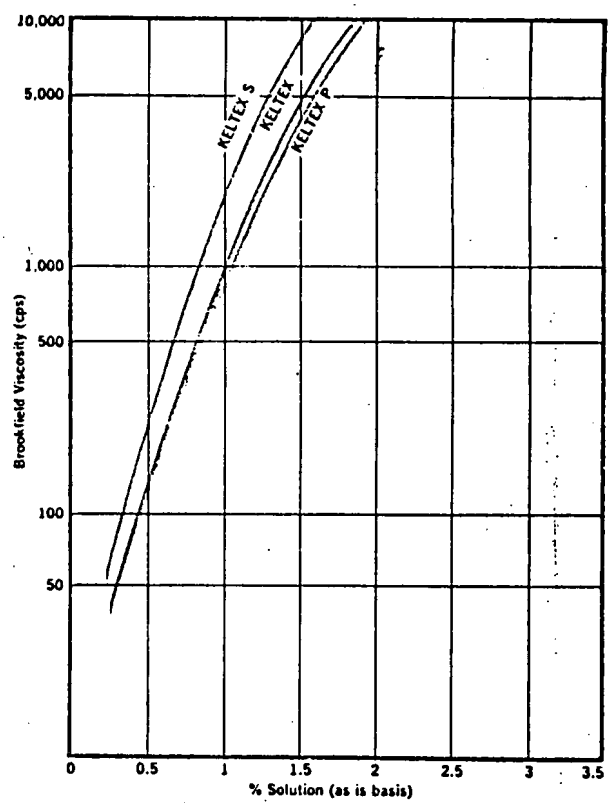
This information is supplied for material-labeling purposes. The analyses are representative and should not be construed as product specifications.

Figure 10-A. Viscosity/concentration curves for refined sodium alginates.



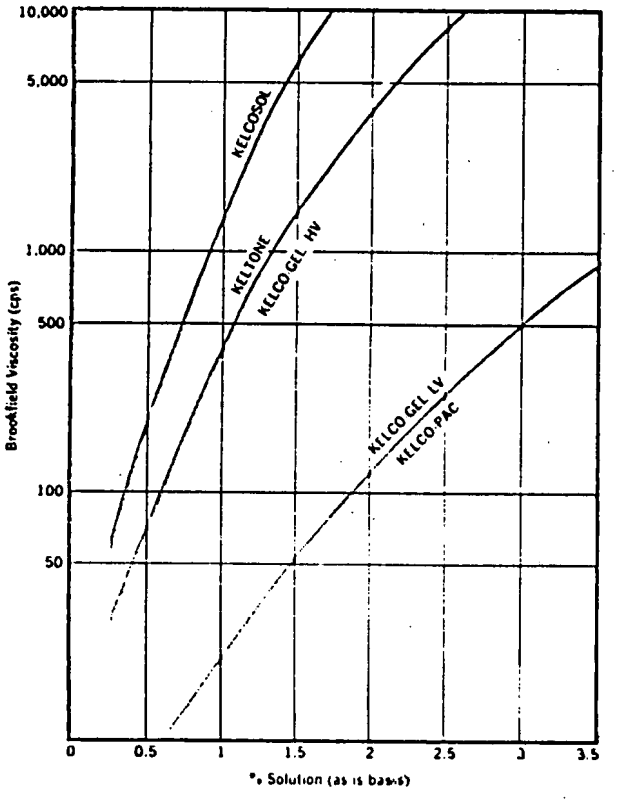
25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Figure 10-B. Viscosity/concentration curves for industrial sodium alginates.



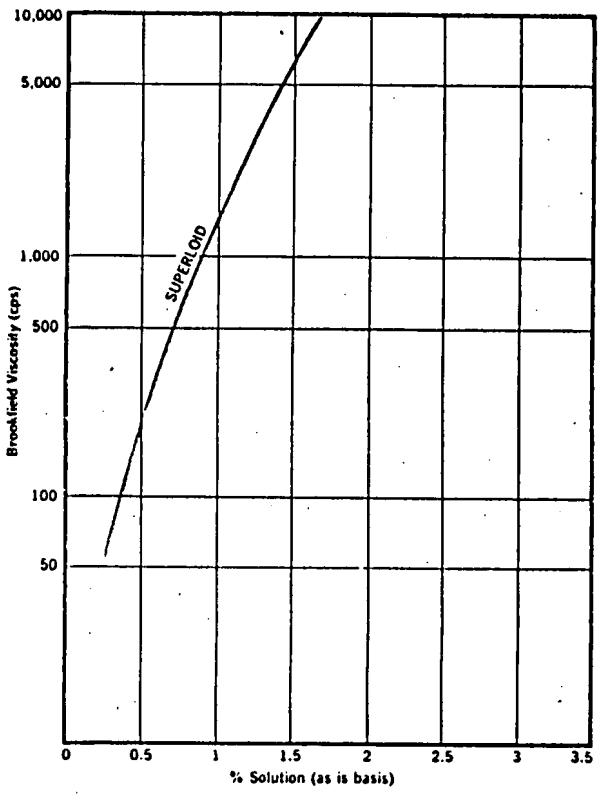
25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Figure 10-C. Viscosity/concentration curves for specially clarified sodium alginates.



25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Figure 10-D. Viscosity/concentration curves for refined ammonium alginate.



25° C. Brookfield LVF Viscometer @ 60 r.p.m.

Table 7
Dry Powder Viscosity Loss
After 1 Year at 75°F

Product	Type	1% Solution Viscosity (cps)* Initial	After 1 Year
KELGIN MV	Sodium Alginate	420	380
KELGIN XL	Sodium Alginate	27	26
KELMAR	Potassium Alginate	270	248
KELTONE	Sodium Alginate	400	330
SUPERLOID	Ammonium Alginate	1,500	675
KELCOLOID HVF	Propylene Glycol Alginate	400	236
KELCOLOID LVF	Propylene Glycol Alginate	115	67

Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle.

General Comments

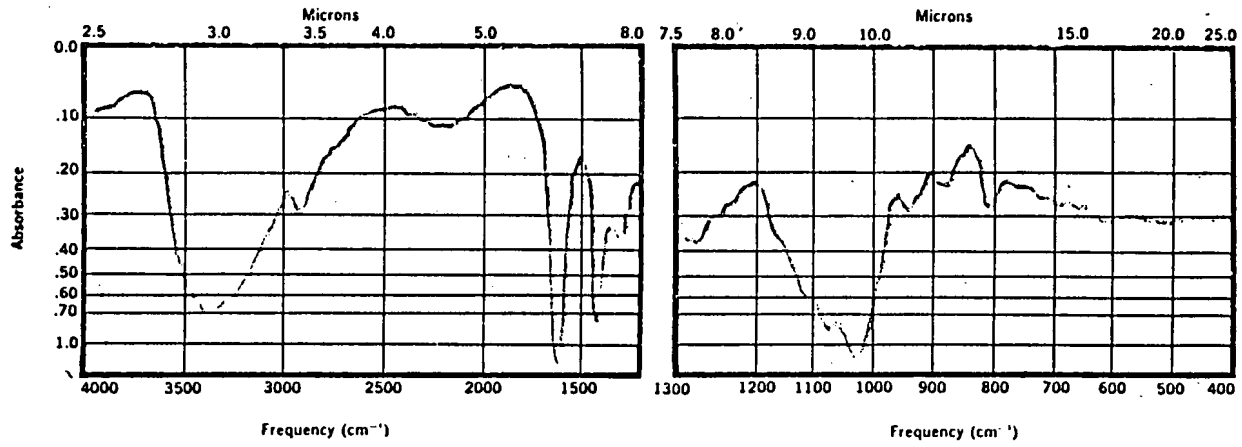
1. High-viscosity alginates usually decrease in viscosity faster than low-viscosity alginate.
2. Ammonium alginate is generally less stable than sodium, potassium, or propylene glycol alginates.
3. Propylene glycol alginates gradually become insoluble when stored at elevated temperatures for extended periods of time.

Table 8
Effect of Storage Temperature
On Viscosity After 1 Year

Product	Type	Storage Temp.	1% Solution Viscosity (cps)* Initial	After 1 Year
KELGIN MV	Sodium Alginate	35°F	420	410
		75°F		380
		90°F		230
KELGIN XL	Sodium Alginate	35°F	27	26
		75°F		26
		90°F		22
SUPERLOID	Ammonium Alginate	35°F	1,500	1,350
		75°F		625
		90°F		300

Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle.

Figure 14. Typical infrared spectrum of a purified sodium alginate obtained from a film.



VII Properties of Algin Solutions

When dissolved in distilled water, pure alginates form smooth solutions having long flow properties. The solution properties are dependent on both physical and chemical variables.

The physical variables which affect the flow characteristics of algin solutions include temperature, shear rate, polymer size, concentration in solution, and the presence of miscible solvents. The effects of shear rate, polymer size, and solution concentration on rheology are examined in detail in Section VIII, "Rheology of Algin Solutions." The effect of polymer size and concentration in solution can be seen in the viscosity/concentration curves in Section V, "Kelco Alginates."

The chemical variables affecting algin solutions include pH, sequestrants, monovalent salts, polyvalent cations, and quaternary ammonium compounds.

Table 9
Viscosity versus Time and Temperature Data in the Absence of Sodium Hexametaphosphate

Hours	77°F Room Temp.	100°F Hot	100°F Room Temp.	130°F Hot	130°F Room Temp.	160°F Hot	160°F Room Temp.	180°F Hot	180°F Room Temp.
			KELGIN HV						
			1% As Is Viscosities in Cps						
0	900	600	800	420	740	360	680	300	
1	900	540	720	400	660	310	600	220	510
2	900	520	700	380	640	290	570	200	480
3	900	500	680	360	620	270	550	180	460
4	900	490	670	350	610	260	530	170	440
			KELGIN MV						
			1% As Is Viscosities in Cps						
0	530	330	400	215	330	185	295	180	290
1	530	290	385	200	310	170	285	160	280
2	530	280	380	195	280	165	275	155	260
3	530	275	370	190	270	160	260	150	250
4	530	260	360	185	265	155	250	145	240
			KELGIN MV						
			1¼% As Is Viscosities in Cps						
0	1000	680	880	450	680	370	625	345	605
1	1000	610	830	410	650	320	600	300	580
2	1000	600	800	400	600	310	570	290	550
3	1000	590	780	390	580	300	550	285	530
4	1000	575	760	380	560	290	535	275	510
			KELGIN LV						
			1% As Is Viscosities in Cps						
0	62	40	54	26	51	22	44	19	40
1	62	38	54	25	49	22	43	19	40
2	62	37	52	25	47	21	42	19	39
3	62	37	52	24	47	20	41	18	38
4	62	36	51	24	46	20	40	18	37
			KELGIN XL						
			1% As Is Viscosities in Cps						
0	40	32	38	22	36	19	34	17	32
1	40	30	36	20	34	18	32	16	30
2	40	28	34	20	32	18	30	16	28
3	40	27	33	19	31	17	29	16	27
4	40	26	32	19	30	17	28	15	26

Physical Variables

A. **Temperature**

Algin solutions, like those of most other polysaccharides, decrease in viscosity with an increase in temperature. Over a limited range, the viscosity of an algin solution decreases approximately 12% for each ten degrees Fahrenheit increase in temperature. Tables 9 and 10 provide data for refined sodium alginates (KELGIN) of four molecular weights. Data were taken for solutions both without and with added sodium hexametaphosphate. Details on the laboratory procedure used in this evaluation follow in item "H" of this section. It is apparent that heating of sodium alginate solutions results in some thermal depolymerization and that the amount of depolymerization is dependent on both time and temperature.

Temperature reduction causes a viscosity increase in an algin solution but does not result in gel formation. A sodium alginate solution which has been frozen and then thawed will not have its appearance or viscosity changed. A freeze-dried sodium-calcium alginate gel can be formed which will have an absorptive capacity of over 5,000 percent but which will be water-disintegrative *(Wise 1972)*.

Table 10
Viscosity versus Time and Temperature Data in the Presence of Sodium Hexametaphosphate
(0.5 gram of Sodium Hexametaphosphate/gram of Algin)

	77°F	100°F		130°F		160°F		180°F	
Hours	Room Temp.	Hot	Room Temp.	Hot	Room Temp.	Hot	Room Temp.	Hot	Room Temp.
				KELGIN HV					
				1% As Is Viscosities in Cps					
0	352	248	310	160	230	130	210	105	200
1	352	235	305	155	225	125	205	100	195
2	352	225	300	150	220	120	200	95	185
3	352	220	295	145	215	115	195	93	182
4	352	215	290	140	210	110	190	90	180
				KELGIN MV					
				1% As Is Viscosities in Cps					
0	155	120	145	65	100	55	100	50	90
1	155	115	130	65	95	55	95	48	86
2	155	108	125	58	90	50	90	44	82
3	155	105	120	55	90	46	85	40	80
4	155	100	115	50	85	42	85	38	76
				KELGIN MV					
				1¼% As Is Viscosities in Cps					
0	330	246	285	115	200	100	190	88	175
1	330	230	270	110	185	95	185	84	165
2	330	215	255	105	175	85	170	75	155
3	330	210	245	100	165	80	160	70	150
4	330	200	235	90	160	75	155	65	140
				KELGIN LV					
				1% As Is Viscosities in Cps					
0	31	22	30	18	29	15	27	13	25
1	31	22	29	18	28	15	26	13	24
2	31	21	29	17	27	14	25	13	23
3	31	21	28	17	26	14	24	12	22
4	31	20	27	16	26	13	23	12	21
				KELGIN XL					
				1% As Is Viscosities in Cps					
0	20	17	20	16	19	15	18	14	17
1	20	17	20	16	19	15	18	14	17
2	20	16	19	15	18	14	17	13	16
3	20	16	19	15	18	14	17	13	16
4	20	16	18	15	18	14	17	13	16

B. Water-Miscible Solvents

Alginates, as hydrophilic colloids, form aqueous solutions. The addition of increasing amounts of non-aqueous water-miscible solvents (alcohols, glycols, acetone, etc.) to an algin solution results in viscosity increases and eventual precipitation. The source of the algin, the degree of polymerization, the cation present, and the concentration in solution all affect the solvent tolerance of the algin solution.

A tabulation of quantities of solvent required for precipitation of algin is given in Table 11.

Chemical Variables

C. Effect of pH

The curves in Figure 15 illustrate the effect of pH variation on the viscosity of solutions of several types of alginates. Sodium alginates with some residual calcium content (KELGIN MV) gel at a pH of 5 and are unstable above a pH of about 11. Sodium alginates with minimal calcium content do not gel until the pH is 3 to 4 (KELCO-GEL LV).

It has been determined that lower-molecular-weight sodium alginates are stable at a pH as low as 3.0 if calcium is completely sequestered. Propylene glycol alginates do not gel until the pH is below 3 but do saponify above pH 6.7.

Although sodium alginate solutions appear to tolerate high pH conditions, long-term stability is poor above about pH 10. At higher pH, β elimination and hydrolysis result in depolymerization with an accompanying viscosity loss.

Figure 15. Effect of pH on algin solutions.

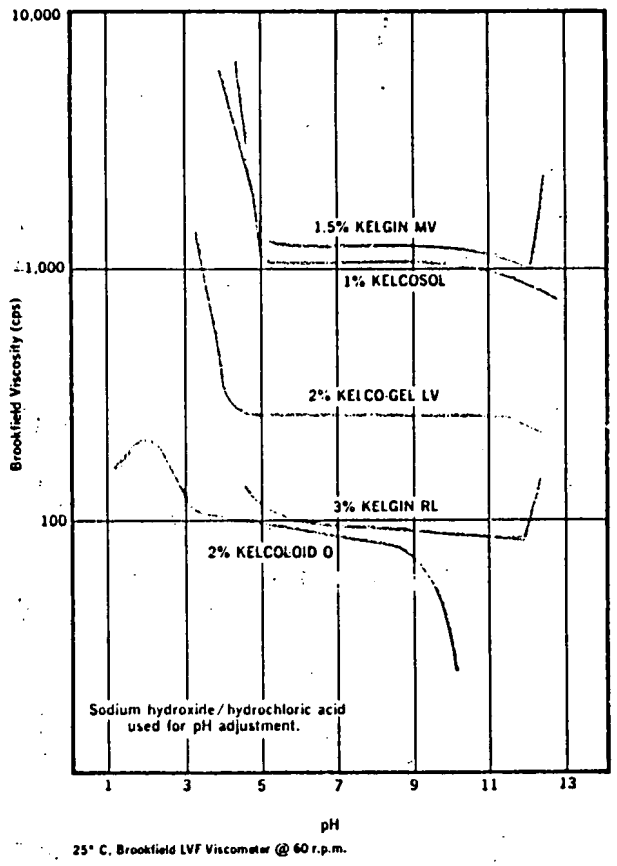


Table 11
Maximum Solvent Tolerance of Algin Solutions

	Methanol	Ethanol	Isopropanol	t-Butanol	Glycerol	Ethylene Glycol	Propylene Glycol	Butyl Cellosolve	Acetone
2% KELGIN XL	20%	20%	10%	20%	70%+	70%+	40%	20%	10%
1% KELGIN MV	20%	20%	10%	10%	70%+	70%+	40%	30%	10%
1% KELGIN HV	20%	20%	10%	20%	70%+	70%+	70%+	20%	10%
1% KELTONE	40%	40%	40%	40%	70%+	70%+	70%+	40%	20%
1% Improved KELMAR	20%	20%	20%	20%	70%+	70%+	50%	40%	20%
1% SUPERLOID	30%	30%	20%	20%	70%+	70%+	70%+	20%	20%
1% KELSET	40%	20%	20%	30%	60%	60%	50%	30%	30%
2% KELCOLOID O	20%	20%	20%	30%	70%+	70%+	50%	30%	10%
1% KELCOLOID D	20%	10%	10%	10%	70%+	70%	40%	40%	10%
1% KELCOLOID LVF	40%	30%	30%	30%	60%	70%+	50%	30%	30%
1% KELCOLOID HVF	30%	20%	20%	20%	60%	70%+	40%	30%	20%

Comments on data:

1. Algin was dissolved in water first, then diluted with solvent to a given water-solvent ratio. Concentration of algin in final solution was 1 percent (except 2 percent for KELGIN XL and KELCOLOID O). Solvent levels were varied in 10 percent increments.
2. The "maximum solvent tolerance" is the solvent percentage next below the percentage at which algin separation was evident.
3. With most solvents, the compatibility end point is quite evident due to algin precipitation and/or viscosity loss. With glycerol and ethylene glycol, however, the end point is not sharp, and apparent compatibility may extend to as high as 90 percent solvent. High solvent combinations are difficult to prepare because the algin must be hydrated prior to addition of the solvent.

Table 14
Algin Compatibility

Material	% Material	% KELGIN MV	Viscosity (cps)* Initial	Viscosity (cps)* After 90 Days
1. Preservatives				
Dowicide® A	0.005	1.0	395	265
	0.1	1.0	350	260
Formaldehyde	0.1	1.0	315	275
	1.0	1.0	325	310
Methyl Parasept®	0.5	1.0	345	285
Vancide® TH	0.1	1.0	275	207
	0.5	1.0	280	202
Sindar® G-4	0.1	1.0	285	222
	0.5	1.0	286	115
Advacide® 340-A	0.1	1.0	392	257
	0.5	1.0	390	268
Omacide® 24	0.1	1.0	375	420
	0.5	1.0	370	650
Surflo® B-17	0.1	1.0	377	320
	0.5	1.0	361	325
Nalco® 248	0.1	1.0	345	192
	0.5	1.0	295	195
Nalco 243	0.1	1.0	346	25
	0.5	1.0	322	227
Metasol® D3T	0.1	1.0	360	217
	0.5	1.0	347	147
Metasol DX3-S	0.1	1.0	332	167
	0.5	1.0	325	125
2. Thickeners				
Xanthan Gum	0.5	0.5	760	730
	1.0	1.0	3,740	4,000
Guar Gum	1.0	1.0	8,320	2,950
Gum Tragacanth	0.5	0.5	350	1,050
	1.0	1.0	5,170	9,150
Methocel® 90HG	0.5	0.5	290	160
Locust Bean Gum	0.5	0.5	340	220
	1.0	1.0	3,950	3,000
3. Water Soluble Resins				
Vinol® PA-20	1.0	1.0	550	350
	5.0	1.0	1,420	230
Carboset® 525	1.0	1.0	480	395
	10.0	1.0	370	280
Carbopol® 934	0.5	0.5	540	100
	1.0	0.5	1,250	650
4. Latex Emulsions				
Rhoplex® AC490	60.0	0.5	170	150
Ucar® 360	60.0	0.5	215	30
Dow Latex 460	60.0	0.5	330	300
Dow Latex 307	60.0	0.5	320	280
Geon® 652	60.0	0.5	280	10
Airflex® 500	60.0	0.5	470	260
Genflo® 355	60.0	0.5	380	405
Genflo 67	60.0	0.5	980	850
5. Organic Solvents				
Acetone	10.0	1.0	590	610
	20.0	1.0	1,710	3,150
Methanol	10.0	1.0	660	680
	20.0	1.0	1,590	3,000
Isopropanol	10.0	1.0	840	970
	20.0	1.0	2,720	4,250
Benzyl Alcohol	10.0	1.0	660	480

*Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle

Table 14 Continued

Material	% Material	% KELGIN MV	Viscosity (cps)* Initial	Viscosity (cps)* After 90 Days
6. Enzymes				
Alkalase*	1.0	1.0	190	155
Cellulase* 4000	1.0	1.0	315	195
Papain	1.0	1.0	323	223
Rhozyme* A-4	1.0	1.0	310	210
Gumase* HP 150	1.0	1.0	290	165
7. Surfactants				
Stepanol* WAT	10.0	0.5	43	37
Igepal* CO 630	20.0	0.5	369	250
Tween* NPX	20.0	0.5	1,290	630
Tergitol* NPX	20.0	0.5	340	220
Miranol* 2 MCA	10.0	0.5	28	38
8. Plasticizers (Glycols)				
Glycerol	50.0	0.5	1,240	3,750
Propylene Glycol	50.0	0.5	325	305
Triethanolamine	50.0	0.5	320	305
Hexylene Glycol	50.0	0.5	990	480
Kromfax*	50.0	0.5	1,010	1,450
Ethylene Glycol	50.0	0.5	1,000	1,120
9. Inorganic Salts				
Ammonium Chloride	1.0	1.0	250	1,100
	5.0	1.0	250	510
	10.0	1.0	230	145
Diammonium Phosphate	1.0	1.0	240	210
	5.0	1.0	210	150
	10.0	1.0	280	180
Ammonium Sulfate	1.0	1.0	235	150
	5.0	1.0	210	60
Magnesium Chloride	1.0	1.0	290	580
	5.0	1.0	130	120
Potassium Chloride	1.0	1.0	210	1,480
	5.0	1.0	210	350
Potassium Phosphate, Dibasic	1.0	1.0	190	170
	5.0	1.0	290	230
Potassium Sulfate	1.0	1.0	290	430
	5.0	1.0	290	230
Sodium Chloride	1.0	1.0	310	880
	5.0	1.0	340	1,090
Sodium Phosphate, Dibasic	1.0	1.0	230	160
	5.0	1.0	215	215
Sodium Sulfate	1.0	1.0	260	360
	5.0	1.0	265	255
Sodium Tetraborate	1.0	1.0	155	175
Sodium Citrate	1.0	1.0	215	170
	5.0	1.0	240	245

Brookfield Model LVF Viscometer at 60 rpm, appropriate spindle

XIII Applications of Kelco Algin Products

Kelco alginates have many applications in foods and industrial products due to their unique properties. The following table illustrates how some of the principal properties apply to typical products:

Food Applications

Property	Product	Performance
Water-holding	Frozen foods	Maintains texture during freeze-thaw cycle.
	Pastry fillings	Produces smooth, soft texture and body.
	Syrups	Suspends solids, controls pouring consistency.
	Bakery icings	Counteracts stickiness and cracking.
	Dry mixes	Quickly absorbs water or milk in reconstitution.
	Meringues	Stabilizes meringue bodies.
	Frozen desserts	Provides heat-shock protection, improved flavor release, and superior meltdown.
	Relish	Stabilizes brine, allowing uniform filling.
Gelling	Instant puddings	Produces firm pudding with excellent body and texture; better flavor release.
	Cooked puddings	Stabilizes pudding system, firms body, and reduces weeping.
	Chiffons	Provides tender gel body that stabilizes instant (cold make-up) chiffons.
	Pie and pastry fillings	Cold-water gel base for instant bakery jellies and instant lemon pie fillings. Develops soft gel body with broad temperature tolerance; improved flavor release.
	Dessert gels	Produces clear, firm, quick-setting gels with hot or cold water.
	Fabricated foods	Provides a unique binding system that gels rapidly under a wide range of conditions.
Emulsifying	Salad dressings	Emulsifies and stabilizes various types.
	Meat and flavor sauces	Emulsifies oil and suspends solids.
Stabilizing	Beer	Maintains beer foam under adverse conditions.
	Fruit juice	Stabilizes pulp in concentrates and finished drinks.
	Fountain syrups, toppings	Suspends solids, produces uniform body.
	Whipped toppings	Aids in developing overrun, stabilizes fat dispersion, and prevents freeze-thaw breakdown.
	Sauces and gravies	Thickens and stabilizes for a broad range of applications.
	Milkshakes	Controls overrun and provides smooth, creamy body.

Industrial Applications

Property	Product	Performance
Water-holding	Paper coating	Controls rheology of coatings; prevents dilatancy at high shear.
	Paper sizings	Improves surface properties, ink acceptance, and smoothness.
	Adhesives	Controls penetration to improve adhesion and application.
	Textile printing	Produces very fine line prints with good definition and excellent washout.
	Textile dyeing	Prevents migration of dyestuffs in pad dyeing operations. (Algin is also compatible with most fiber-reactive dyes).
Gelling	Air freshener gel	Firm, stable gels are produced from cold-water systems
	Explosives	Rubbery, elastic gels are formed by reaction with borates.
	Toys	Safe, nontoxic materials are made for impressions or putty-like compounds.
	Hydro-mulching	Holds mulch to inclined surfaces; promotes seed germination.
	Boiler compounds	Produces soft, voluminous flocs easily separated from boiler water.
Emulsifying	Polishes	Emulsifies oils and suspends solids.
	Antifoams	Emulsifies and stabilizes various types.
	Latices	Stabilizes latex emulsions, provides viscosity.
Stabilizing	Ceramics	Imparts plasticity and suspends solids.
	Welding rods	Improves extrusion characteristics and green strength.
	Cleaners	Suspends and stabilizes insoluble solids.

A comparative study of the cytotoxicity of silver-based dressings in monolayer cell, tissue explant, and animal models

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ABSTRACT

Over the past decade, a variety of advanced silver-based dressings have been developed. There are considerable variations in the structure, composition, and silver content of these new preparations. In the present study, we examined five commercially available silver-based dressings (Acticoat™, Aquacel® Ag, Contreet® Foam, PolyMem® Silver, Urgotul®SSD). We assessed their cytotoxicity in a monolayer cell culture, a tissue explant culture model, and a mouse excisional wound model. The results showed that Acticoat™, Aquacel® Ag, and Contreet® Foam, when pretreated with specific solutes, were likely to produce the most significant cytotoxic effects on both cultured keratinocytes and fibroblasts, while PolyMem® Silver and Urgotul®SSD demonstrated the least cytotoxicity. The cytotoxicity correlated with the silver released from the dressings as measured by silver concentration in the culture medium. In the tissue explant culture model, in which the epidermal cell proliferation was evaluated, all silver dressings resulted in a significant delay of reepithelialization. In the mouse excisional wound model, Acticoat™ and Contreet® Foam indicated a strong inhibition of wound reepithelialization on the postwounding-day 7. These findings may, in part, explain the clinical observations of delayed wound healing or inhibition of wound epithelialization after the use of certain topical silver dressings. Caution should be exercised in using silver-based dressings in clean superficial wounds such as donor sites and superficial burns and also when cultured cells are being applied to wounds.

The use of silver as an antimicrobial agent has a long history.[1] Its recent resurgence follows from Moyer's use of silver nitrate solution in patients with burns.[2] Solutions gave way to cream formulations but it is now silver-containing dressings that provide the widest range of silver-based wound care products.[3,4] Despite the ever increasing number of commercially available silver-based dressings, there is a distinct lack of comparative data on their clinical effectiveness.[5] What is known is that silver can be effective against a wide range of microorganisms, including aerobic, anaerobic, Gram-negative and Gram-positive bacteria, yeast, fungi, and viruses. The antimicrobial effect of silver can be explained by various mechanisms: silver interferes with the respiratory chain in the cytochromes of microbacteria; additionally, silver ions also interfere with components of the microbial electron transport system, bind DNA, and inhibit DNA replication.[6,7]

Dressings are designed to have more controlled and prolonged release of silver during the entire wear-time when compared with the cream formulations. This allows dressings to be changed less frequently, thereby reducing risk of nosocomial infection, cost of care, further tissue damage, and patient discomfort. Many factors affect the clinical performance of a dressing. The amount of silver content, the chemical, and physical forms of the silver, silver distribution, and even the affinity for moisture all participate in a dressing's capability to exert a significant antimicrobial effect. Marked differences exist in a variety of silver dressing products. These products can be categorized as: (i) silver-delivery dressings such as Acticoat™ (Smith & Nephew, Hull, UK) and Urgotul®SSD (Laboratory URGD, Chenove, France), which have silver content coated (on the surface) or impregnated into the dressing material. They deliver silver to the wound site after direct contact; (ii) silver-containing dressings such as Aquacel®Ag (Cenvatec, Deeside, UK), Contreet® Foam (Coloplast, Humlebaek, Denmark), and PolyMem® Silver (Ferris Mfg. Corp., Burr Ridge, IL), which have high absorptive capacities and "lock up" the silver content until the dressing absorbs wound exudate or moisture. In

DMEM	Dulbecco's modified Eagle media
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGM	Fibroblast growth medium
ICP-MS	Inductively coupled plasma mass spectrophotometer
KGM	Keratinocyte growth medium
K-SFM	Keratinocyte serum-free medium
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
PWD	Postwounding day

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Exhibit M

Urgotul® SSD, the silver is in the form of silver sulfadiazine, which is released into the wound bed. This is metabolized to release silver and sulfadiazine moeties.[8] Traditionally, the active silver agent has been thought to be ionic silver but in the nanocrystalline form, elemental silver is also thought to be active.[9] Independent studies involving comparisons between different types of dressings suggested considerable variations in one or more aspects of dressings' performance.[4,9–11] Our published microbiology data demonstrated that Acticoat™ and Contreet® Foam have a broad spectrum of bactericidal activities against both Gram-positive and -negative bacteria, and Contreet® Foam was characterized by a very rapid bactericidal action.[12] However, we and others have also demonstrated in vitro cytotoxic effects of silver nitrate ($AgNO_3$), silver sulfadiazine, and Acticoat™ on cultures of keratinocytes and fibroblasts.[13–15]

In the present study, we are concerned with the cytotoxicity of a range of commercially available silver dressings to the viable cells in the wound bed. We have examined the biological effects of these dressings on: first, isolated skin cells—the monolayer culture of human keratinocytes and fibroblasts; second, the tissue explant culture model—pig mid-dermis culture for epidermal cell proliferation; and last, the mouse excisional wound model.

MATERIALS AND METHODS

Materials

Five silver-based dressings (Acticoat™, Aquacel® Ag, Contreet® Foam, PolyMem® Silver, Urgotul®SSD) and one control dressing without silver (Aquacel®) were obtained from commercial sources. The components of these dressings are summarized in Table 1.

Human keratinocyte and fibroblast cultures were developed from primary cultures of discarded surgical tissues or foreskins, according to Institution Ethical guidelines. 3T3/NIH fibroblasts were obtained from the American Type Culture Collection (ATCC; Rockville, MD). All the media and reagents used in cell cultures were purchased from GIBCO (Grand Island, NY) unless specified.

Six- to 8-month-old large white pigs and C57 BL/6J mice were sourced from the Laboratory Animal Services Centre of the Chinese University of Hong Kong (CUHK). All animal procedures were subject to the approval of the Animal Experimentation Ethics Committee of the CUHK, and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Keratinocyte culture

Keratinocytes were isolated from the skin tissues of discarded surgical tissues or foreskins as previously described.[15] Briefly, surgical samples were collected in skin transport medium comprising Dulbecco's modified Eagle media (DMEM) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL Fungizone® (Invitrogen, Grand Island, NY), and 50 µg/mL gentamicin. The skin biopsy was transferred to the laboratory and trimmed by a surgical blade to remove all adipose tissue and cut into 2–3 mm wide strips before immersion in 2 mg/mL dispase II (Roche Diagnostics,

Table 1. Dressings

Dressing Name	Manufacturer	Basic dressing composition	Silver composition	Silver release
AQUACEL®	ConvaTec (Deeside, UK)	Hydrocolloid fiber (sodium carboxymethylcellulose)	None	—
AQUACEL® Ag	ConvaTec	Hydrocolloid fiber (sodium carboxymethylcellulose)	1.2% w/w ionic silver (silver nitrate)	Ag^+
Acticoat™	Smith & Nephew (Hull, UK)	An absorbent polyester inner core sandwiched between two outer layers of silver-coated polyethylene net	Metallic nanocrystalline silver	Ag^0
Contreet® Foam	Coloplast (Humlebaek, Denmark)	Polyurethane foam	Ionic silver (silver sodium hydrogen zirconium phosphate)	Ag^+
PolyMem® Silver	Ferris Mfg. Corp. (Burr Ridge, IL)	Polyurethane foam containing a safe nontoxic cleanser (F-68 surfactant), a moisturizer (glycerol) and an absorbing agent (superabsorbent starch copolymer)	Elemental nanocrystalline silver (124 ug/cm^2)	Ag^0
Urgotul® S.S.D	Laboratory URGO (Chenove, France)	Polyester gauze dressing impregnated with hydrocolloid particles dispersed in a Vaseline paste	Silver sulfadiazine	Ag^+

Basel, Switzerland) in DMEM for overnight digestion at 4 °C. The following day, the epidermis was mechanically separated from the dermis and collected into 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA) solution. Basal keratinocyte cells were dissociated by vortex for 1 minute and neutralized with three volumes of DMEM with 10% FBS. The cells were then pellected by spinning at 200×*g* for 5 minutes. Cells were resuspended in keratinocyte growth medium (KGM) consisting of one volume of Ham's F12, three volumes of DMEM, 10% FBS, 100 U/mL penicillin, 100 μL/mL streptomycin, 0.25 μg/μL amphotericin B, 100 fM cholera enterotoxin, 5 μg/mL transferring, 180 μM adenine sulfate, 5 μg/mL insulin, 10 mg/mL epidermal growth factor, 0.4 μg/mL hydrocortisone (Sigma), and 20 fM liothyronine (Sigma). The cell suspension was seeded onto 4 μg/mL mitomycin C-treated-3T3/NIH feeder layer at a density of $3\times10^4/cm^2$. Keratinocytes were subcultured at 70–80% confluence after differential dissociation of the feeder layer with 0.02% EDTA for 10 minutes, followed by keratinocyte cell dispersion with 0.05% trypsin-0.02% EDTA treatment for 5 minutes. Cells were expanded one more passage on feeder cells and stored under liquid nitrogen in KGM with 5% dimethyl sulfoxide (DMSO) and 20% FBS. Cells at passages 3–5 were used in the following experimental assays.

Fibroblast culture

The dermis was separated from the skin tissues of discarded surgical tissues or foreskins by dispase digestion as previously described.[15] The dermis was then finely minced, and resultant cell suspension, together with small pieces of tissues, were transferred to culture dishes and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μL/mL streptomycin, and 0.25 μg/μL amphotericin B (fibroblast growth medium, FGM). Medium was changed weekly. Upon reaching confluence, cells were passaged using 0.25% trypsin-EDTA. Cells at passages 4–10 were used in the following experimental assays.

Dressing absorbency test

The dressing absorbency test was performed according to the instructions from the "Test methods for primary wound dressings-Part 1: Aspects of absorbency" issued by the State Food and Drugs Administration, People's Republic of China, which is compatible with the European reference of Test methods for primary wound dressings—the EN 13726:2002 – section 3.2 free-swelling capacity. In brief, the testing dressing was cut into $1\times1\ cm^2$ size and was weighed (W_0). A solution A composed of 142 mmol Na ions and 2.5 mmol Ca ions was first prewarmed to 37 °C, and then the testing dressing as well as solution A were placed onto a 100 mm Petri dish and incubated at 37 °C for 30 minutes. The volume of solution A required was 40 times larger than the weight of the dressing. The dressing was weighed again after a 30-minute incubation (W_{30}). The absorbency of the dressing was expressed as volume of solution A absorbed per square centimeter dressing ($[W_{30}–W_0]$/area) and set as one of the parameters for dressing pretreatment as described below.

Pretreatment of dressing materials

All dressings were cut into $1\times1\ cm^2$ size under a sterile condition. Based on the dressing absorbency test result, PolyMem® Silver was found to have the highest absorbency of $0.8\ mL/cm^2$. The time for the foam dressings to be fully saturated with solution A was 10 minutes. Therefore, for the pretreatment, all the dressings were soaked with 0.8 mL of different solutes—deionized water, saline, and 100% FBS, respectively, for 10 minutes at 37 °C. The dressings, as well as individual pretreatment solutions, were then added to the culture for cytotoxicity assay.

Cytotoxicity testing of dressing materials on keratinocyte and fibroblast monolayer cultures

The cytotoxicity testing of six types of dressings on both keratinocyte and fibroblast monolayer cultures was performed in the same manner, except for using different culture medium and cell densities. Briefly, keratinocytes were seeded into six-well plates at a density of 1×10^5/well and cultured in defined keratinocyte serum-free medium (K-SFM; GIBCO)-containing insulin, epidermal growth factor, and fibroblast growth factor. Fibroblasts were seeded at a density of 5×10^4/well in FGM. Upon 3–4 days culture until 70–80% cell confluence, the $1\times1\ cm^2$ dressings were presoaked with 0.8 mL of deionized water, saline, or FBS, respectively, as mentioned above. Afterward, the dressings together with individual pretreatment solutions, and 2.2 mL of culture medium, were added to each of the culture wells. Addition of 0.8 mL of the plain solute without dressing being soaked was regarded as a positive control. The cells were then incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 2, 4, 6, and 24 hours. At each time point, dressings were removed and cell viability was determined by an 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Experiments were performed in triplicate.

MTT assay

MTT is a yellow dye that is taken up by viable cells and converted to Formazen 8 (purple crystals). Change in color can be assessed spectrophotometrically to give an assessment of metabolic activity as a function of cytotoxicity.[15] At each time point of the cytotoxicity testing, dressings from each well were removed and culture medium was aspirated. One milliliter MTT solution (0.5 mg/mL) in complete culture medium was added to each cell-containing well and then incubated in a humidified atmosphere for 2–4 hours in the dark. Afterward, 1 mL solubilization solution (10% SDS in 0.01 M HCl) was added and the plates were maintained at 37 °C in an incubator in a humidified atmosphere overnight. Afterward, 200 μL of the solubilization solution from each well was transferred to a 96-well enzyme-linked immunosorbent assay (ELISA) plate for spectrophotometric measurement using a microplate (ELISA) reader (Spectra Rainbow, TECAN, Grödig, Austria), with a test wavelength of 570 nm and a reference wavelength of 690 nm.

The results of the MTT assay were expressed as the relative cell viability of individual experimental treatment to that of the control group at 2 hours (mean ± SD). The

statistical significance was assessed by Student's *t*-test using Prism 3.0 software from Graphpad, and $p < 0.05$ was considered to be significant.

Silver content of dressing materials

Samples of each dressing in $1 \times 1\,cm^2$ were digested by heating in a mixture of concentrated HNO_3 and HCl to break down the dressing matrix and to release and dissolve all of the silver present. The digest was then filtered and diluted with deionized water. Total silver in the aqueous samples was determined by inductively coupled plasma mass spectrometry (ICP-MS, 7,500c, Agilent Technologies Inc., Palo Alto, CA). Sample aerosol generated by a neubilizer was carried to an argon plasma of about 8,000 K for the production of silver ions. The sample ion was then introduced into a mass spectrometer for ion identification and quantification. For verification of the accuracy of the analyte, a standard reference material (SRM 1,577b, Bovine Liver, the National Institute of Standards and Technology, Gaithersburg, MD) was analyzed along with the samples. The sensitivity of the assay was 1.6 nmol/L. The interassay coefficients of variations were 3.7% at 38 nmol/L and 2.5% at 320 nmol/L. The total extractable silver content of each dressing was then determined and expressed in $\mu g/cm^2$.

Silver dissociation of dressing materials in different solution

To determine silver dissociation of each dressing in different solutions, dressing samples in $1 \times 1\,cm^2$ piece were presoaked with 0.8 mL of deionized water, saline or 100% FBS, respectively, for 10 minutes and forwarded to 2.2 mL culture medium, K-SFM, or FGM, respectively, for incubations at 37 °C for 2, 4, 6, and 24 hours. The extraction solution was then centrifuged at 940g for 10 minutes at 4 °C, and 200 μL of each supernatant was collected for ICP-MS assay as described above. The total amount of silver released over time was then determined and expressed in microgram per liter (μg/mL).

Pig mid-dermis explant culture

This model is based on a previously described technique.[16] Briefly, mid-dermal sheets from the paravertebral areas of 6–8-month-old large white pigs were harvested under aseptic conditions using a Pagett's dermatome at a setting of 0.5 mm after removing a 0.5 mm thick split-thickness graft containing the epidermis. Dermal strips were cut into $1 \times 1\,cm^2$ segments and transferred onto sterile dressing gauze in Petri dishes to raise the explant to the air–liquid interface for culture. Orientation of the dermis was maintained. Explants were maintained in 5% CO_2 environment with 95% humidity in serum-free DMEM supplemented by 10 ng/mL hydrocortisone. The testing dressings of size $1 \times 1\,cm^2$ each were laid on the surface of the explants on day 0. The cultures were harvested at days 4 and 8, and subject to formalin fixation and rhodamine staining for visualization of the reepithelialized areas around the hair follicles. Resurfaced areas were photographed and their size was determined using computerized morphometric analysis (Metamorph 4.0). The ratio of area of reepithelialization to the number of hair follicles in a certain area was determined to give an index of reepithelialization.

Mouse excisional wound model

This model is based on that described by Galiano et al.[17] Four full-thickness excisional wounds extending through the panniculus carnosus were created on each C57 BL/6J mouse using a 6-mm diameter biopsy punch. A silicone splint was added onto each wound and sutured to reduce the contraction effect during wound closure. The wounds were either dressed with testing dressings or not dressed (control), followed by a cover of Tegaderm (a semi-occlusive dressing) to hold the dressings in place. At two time-points, post-wounding day 4 (PWD 4) and 7 (PWD 7), the whole-wound tissues were dissected and subjected to standard histological examination of the reepithelialization. The epithelial gap (EG) and wound gap (WG) of each wound were measured at microscopic level using image analysis software. The percentage of EG/WG (EGW) for each wound was then calculated as an indicator of wound-healing rate.

RESULTS

In vitro cytotoxicity of silver-based dressings on cultured human keratinocyte

As shown in Figure 1, the relative cell viability of keratinocytes after different dressing treatments varied significantly.

In control treatments in which three types of plain solute without dressing being soaked was individually added (see "Materials and Methods"), addition of serum (FBS) but not water and saline markedly suppressed the proliferation of keratinocyte. Addition of 0.8 mL of FBS in total volume of 3 mL culture medium led to a 30% reduction of cell viability at 24 hours when compared with that at 2 hours.

Aquacel®, as a control dressing without silver, showed mild cytotoxicity on keratinocytes when delivered by water and saline. About 88 and 75% viable keratinocytes remained, respectively, after 24 hours dressing delivery by water and saline, respectively. When delivered in FBS, no additional reduction of cell viability was obviously observed compared with the control treatment.

Acticoat™ exerted a severe cytotoxic effect on keratinocytes when delivered by water. Eighty percent of cells were killed immediately after the dressing was delivered with water for 2 hours. After 24 hours only $1.6 \pm 0.33\%$ viable cells remained. However, by using saline as a pretreatment solute, cell viability was significantly improved and remained around 80% during 24 hours. On the other hand, FBS pretreatment also improved cell viability but still led to 50% cell death after 24 hours.

Aquacel® Ag was cytotoxic to keratinocytes in all three pretreatment statuses. The relative cell viability at 24 hours was $53.1 \pm 5.04\%$, $9.0 \pm 5.80\%$, and $19.9 \pm 11.53\%$, respectively, when the dressing was delivered by water, saline, and FBS pretreatment, respectively.

Contreet® Foam was severely cytotoxic to keratinocytes when delivered by FBS. More than 95% cells were killed immediately after the dressing was delivered with FBS for 2 hours and the killing effect lasted till 24 hours. Using water and saline as the pretreatment solutes, this

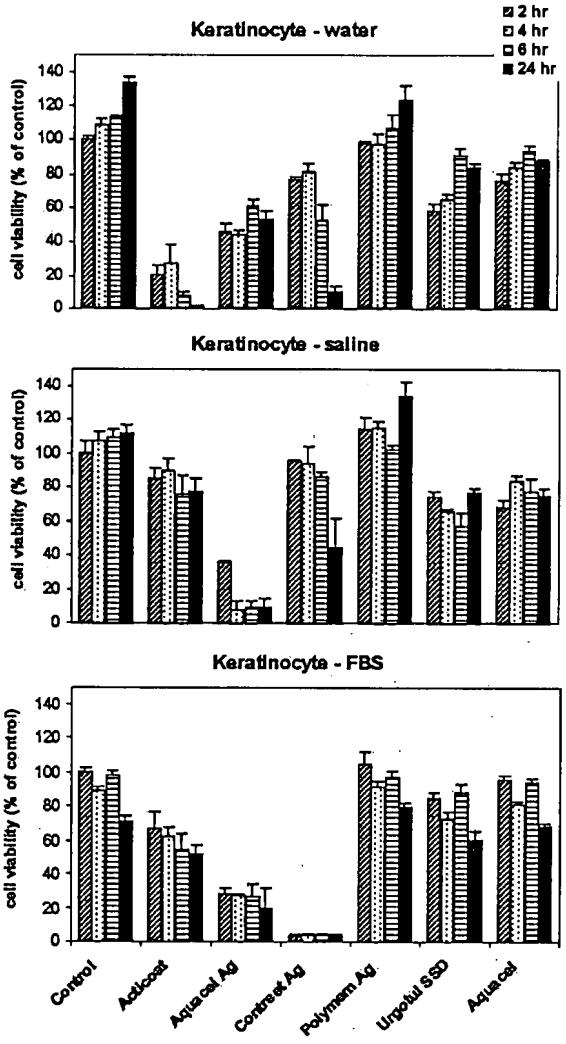

Figure 1. The relative cell viability of keratinocytes after various dressing treatments for 2, 4, 6, and 24 hours was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dressings were pretreated with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). Experiments were performed in triplicate. The results are expressed as percentage of the cell viability of individual experimental treatment to that of the control group at 2 hours (mean ± SD).

dressing did not promote a significant cell death within a short period but led to 90 and 50% cell death, respectively, at 24 hours.

PolyMem® Silver was relatively safe for keratinocytes in all three pretreatment statuses. No obvious cytotoxic effect was observed in comparison with individual controls. In fact, the dressing was shown to enhance keratinocyte proliferation slightly when delivered by saline.

Urgotul®SSD was also relatively safe for keratinocytes. Its biological activity on the growth of keratinocyte was comparable with that of the nonsilver-containing dressing, Aquacel®.

In brief, PolyMem® Silver was shown to be relatively safe for cultured keratinocytes, while Acticoat™, Aquacel® Ag, and Contreet® Foam were significantly lethal when delivered by specific pretreatment solute.

In vitro cytotoxicity of silver-based dressings on cultured human fibroblast

As shown in Figure 2, the response of cultured fibroblasts to different dressing treatments also varied significantly.

In control treatments, the growth of fibroblasts was not significantly affected by the addition of the plain pretreatment solutes, water, and saline. While fibroblasts responded differently from keratinocytes to the addition of serum solute (FBS): no cell growth inhibition, rather a doubled increase of fibroblast proliferation, was observed within 24 hours.

Aquacel®, as a nonsilver-containing dressing, hardly affected the cell growth of the fibroblast no matter which pretreatment solute was used. Within 24 hours of dressing delivery, fibroblasts proliferated similarly as they did in the control treatments.

Acticoat™ exerted a significant cytotoxic effect on fibroblasts when delivered by water. Seventy percent of cells were killed immediately after the dressing was delivered with water for 2 hours, and after 24 hours, only 25.2 ± 0.78% viable cells remained. By using saline as a pretreatment solute, the cell viability of fibroblast was improved but still remained less than 50% at 24 hours. However, the cell viability was improved to more than 70% at 24 hours when the dressing was delivered with FBS.

Aquacel® Ag exerted severe cytotoxicity on fibroblasts no matter which pretreatment solute was used. The lethal effect was observed immediately after 2 hours of dressing delivery. Less than 25% fibroblasts could survive after being exposed to Aquacel® Ag for 24 hours.

Contreet® Foam was also severely cytotoxic to fibroblasts after 24-hour delivery. Among the three pretreatment solutes, a relatively slow onset of action was observed when the dressing was delivered by saline pretreatment, 63.7 ± 1.87% cells remained after 2 hours, while 60.1 ± 5.17, 46.7 ± 8.36, and 22.9 ± 3.31% viable cells remained after 4, 6, and 24 hours, respectively.

PolyMem® Silver was observed to show mild cytotoxicity to fibroblasts within a short period. At 6 hours of the dressing delivery, about 77.5 ± 0.00, 63.1 ± 3.18, and 82.8 ± 1.21% viable cells remained, respectively, in water-, saline-, or FBS-delivered treatments. After 24 hours, the cells seemed tolerant to all the treatments and started to proliferate again.

Urgotul®SSD affected fibroblast in a manner similar to PolyMem® Silver. Using saline as the pretreatment solute, around 30% cells were killed within 6 hours of the dressing delivery, while the cells were observed to proliferate again at 24 hours.

In brief, PolyMem® Silver and Urgotul®SSD were the least cytotoxic to cultured fibroblasts, while Acticoat™, Aquacel® Ag, and Contreet® Foam showed significant killing or inhibitory effect on the cell growth of the fibroblast.

Silver content and silver dissociation of the dressings

The measured total silver content of the dressings is shown in Table 2, which indicates large differences between the

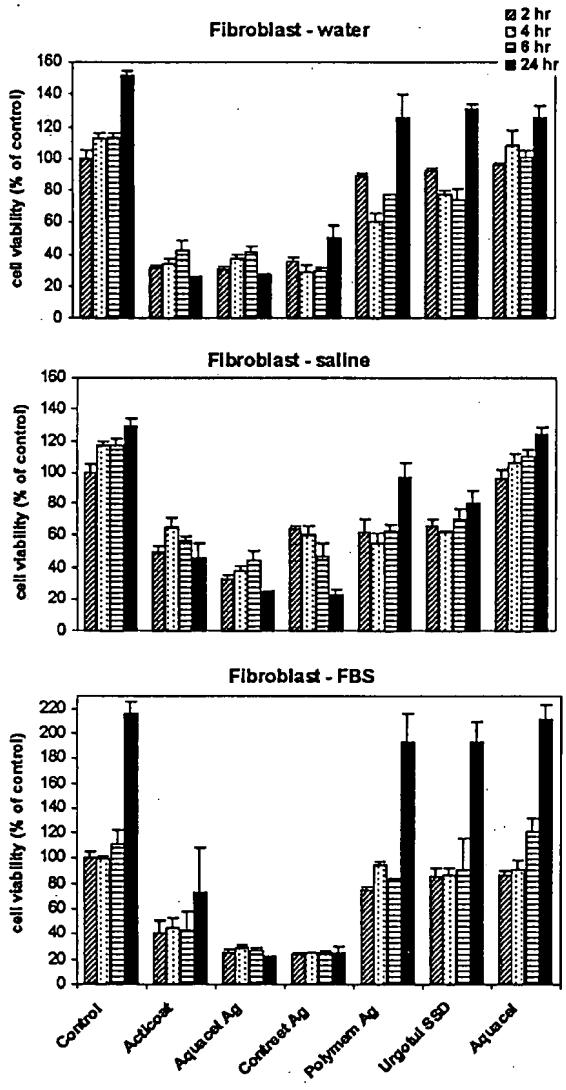

Figure 2. The relative cell viability of *Fibroblasts* after various dressing treatments for 2, 4, 6, and 24 hours was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dressings were pretreated with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). Experiments were performed in triplicate. The results are expressed as percentage of the cell viability of individual experimental treatment to that of the control group at 2 hours (mean ± SD).

five products, ranging from 13 μg for Contreet® Foam to 934 μg for Acticoat™ for a 1 cm^2 sample.

The amount of silver released into different culture medium including keratinocyte culture medium (K-SFM serum-free medium) and fibroblast growth medium (FGM—DMEM+10% FBS) after the dressing was presoaked with different solutes including water, saline, or FBS, also varied extensively over time. An increase in silver release was seen with the increase of the time. It is also noteworthy that, when the dressings were presoaked with

Table 2. Silver content of the dressings

	Ag content (μg/cm^2)
Acticoat™	934
Aquacel® Ag	21
Contreet® Foam	13
PolyMem® Silver	139
Urgotul®SSD	85

FBS, the amount of silver released into either keratinocyte culture medium or fibroblast growth medium was significantly improved, in particular, in the case of Acticoat™, Aquacel® Ag, and Contreet® Foam (Figures 3 and 4).

Comparison of the silver release and silver content for different dressings revealed no correlation. Comparison of the in vitro cytotoxicity and silver dissociation in different pretreatment solutes and culture mediums revealed that, generally, there was an obvious correlation of these two factors for different dressings (Figures 3 and 4). For example, when compared with Acticoat™, Aquacel® Ag, and Contreet® Foam, PolyMem® Silver, and Urgotul®SSD, two silver-containing dressings that showed the least cytotoxicity to both cultured keratinocytes and fibroblasts, released much less amount of silver into the culture medium over time. However, such a correlation was not always exactly consistent for highly cytotoxic dressings such as Acticoat™, Aquacel® Ag, and Contreet® Foam. For example, in keratinocyte cultures, when presoaked with saline, Contreet® Foam showed the largest silver release at 24 hours (Figure 3D). Aquacel® Ag, however, not Contreet® Foam, was found to be the most cytotoxic under the same conditions (Figure 1).

In situ cytotoxic effect of silver-based dressings on epidermal reepithelialization

The cytotoxic effects of silver-based dressings on an epidermal cell proliferation model—pig mid-dermis explant culture—were also examined. As shown in Figure 5A, in the control cultures, the reepithelialization index (reepithelialization area [mm^2] per hair follicle) kept growing from days 0 to 8, while a delayed reepithelialization was observed in all explant cultures covered by silver-based dressings. The least number and area of rhodamine-stained reepithelialized surface was seen in the Acticoat™-dressed explant culture, whose reepithelialization index was 0.083 ± 0.005 and 0.089 ± 0.014, respectively, at days 4 and 8. Histological examination (Figure 5B) also confirmed the significant reepithelialization in the control group, but not in samples treated with silver-based dressings.

In vivo cytotoxicity effects of silver-based dressings on wound reepithelialization

We used a mouse excisional wound model to study the effect of silver-based dressings on wound epidermal reepithelialization. As shown in Figure 6A, at the microscopic

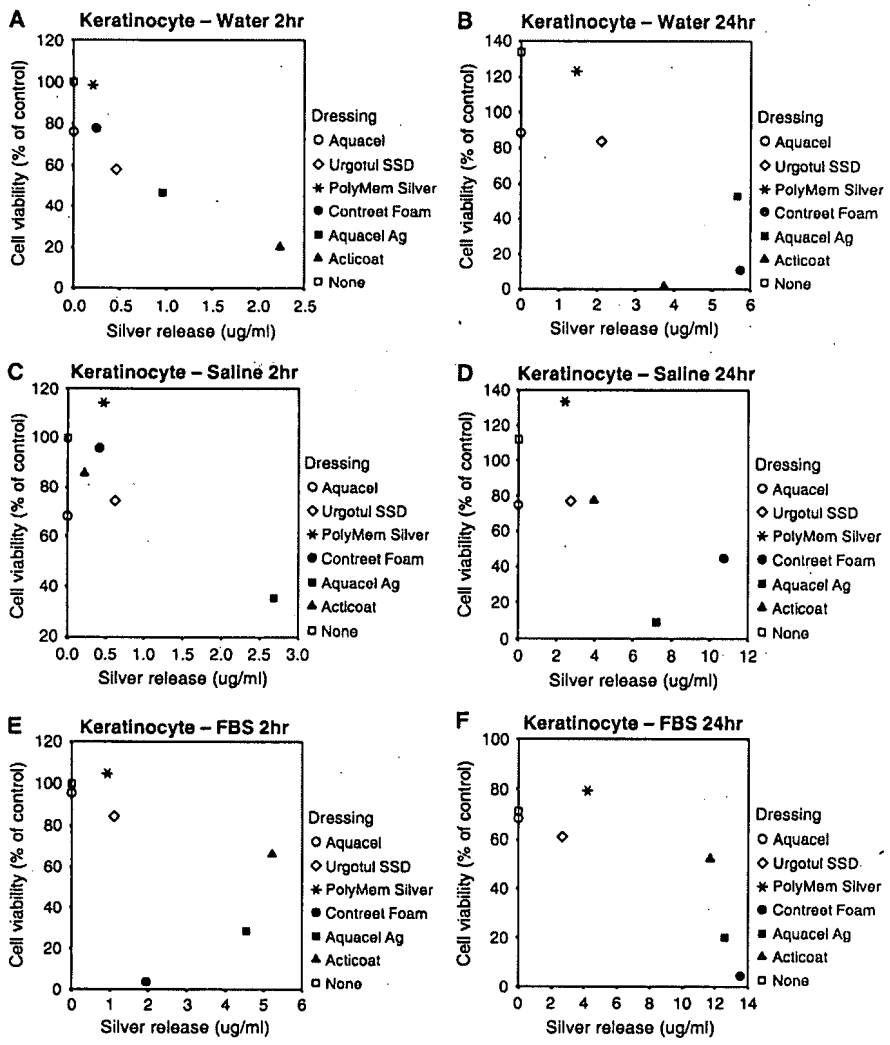

Figure 3. Comparison of the amount of silver released (ug/mL) into keratinocyte culture medium with the relative cell viability (%) after various dressing treatments for 2 and 24 hours. The dressings were presoaked with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). The total amount of silver released into the culture medium over time was determined by an inductively coupled plasma mass spectrophotometer (ICP-MS) assay. A scatter diagram was plotted to analyze the relationship between the silver concentration and the cell viability.

level, the epithelial tongue (ET) comprised keratinocytes growing from the adjacent unwounded epidermis toward the center of the wound. The epithelial gap (EG) and wound gap (WG) of each wound were measured; the percentage of EG/WG (EGW) for each wound was then calculated as an indicator of wound-healing rate (the larger the EGW, the more the delay in reepithelialization). The data are summarized in Figure 6B. As shown, the inhibitory effect of silver-based dressings was not significant on PWD4. On PWD7, Acticoat™, and Contreet® Foam indicated a strong inhibition of reepithelialization, the EGW of which were 71.4 and 73.1%, respectively; in comparison, the EGW of the control group was 25.9% only on PWD7. Aquacel® Ag, and PolyMem® Silver also impaired wound reepithelialization on PWD.[7]

DISCUSSION

The results from this study serve to underline the complex differences between a small range of commercially available dressings. At the outset, we sought to establish a "battery" of laboratory-based models which could be used to assess the potential clinical performance of the ever-rapidly proliferating range of silver-based dressings. Cell culture techniques are well established and highly reproducible and have been used by others and ourselves to assess silver toxicity.[15,18] There are limitations, however, to using monolayer cultures eloquently described by Lansdown as "naked cells" in a review of silver in wound care.[19] In our own clinical experience, we can acknowledge the value of hypochlorite solution in wound care which fell out of favor in the "Eusol" debate.[15] Thus, in this evaluation, we sought to extend the models and add to the biological complexity by using a three-dimensional tissue explant model and an animal model, albeit murine, of dynamic epidermal cell proliferation. It is also relevant to observe that in our clinical practice, the major focus is on healing wounds where infection is more a feature to be prevented, than treated, and unimpeded cell proliferation is the biological means to the clinical end point of wound closure. Having said that, the antimicrobial effect of the silver

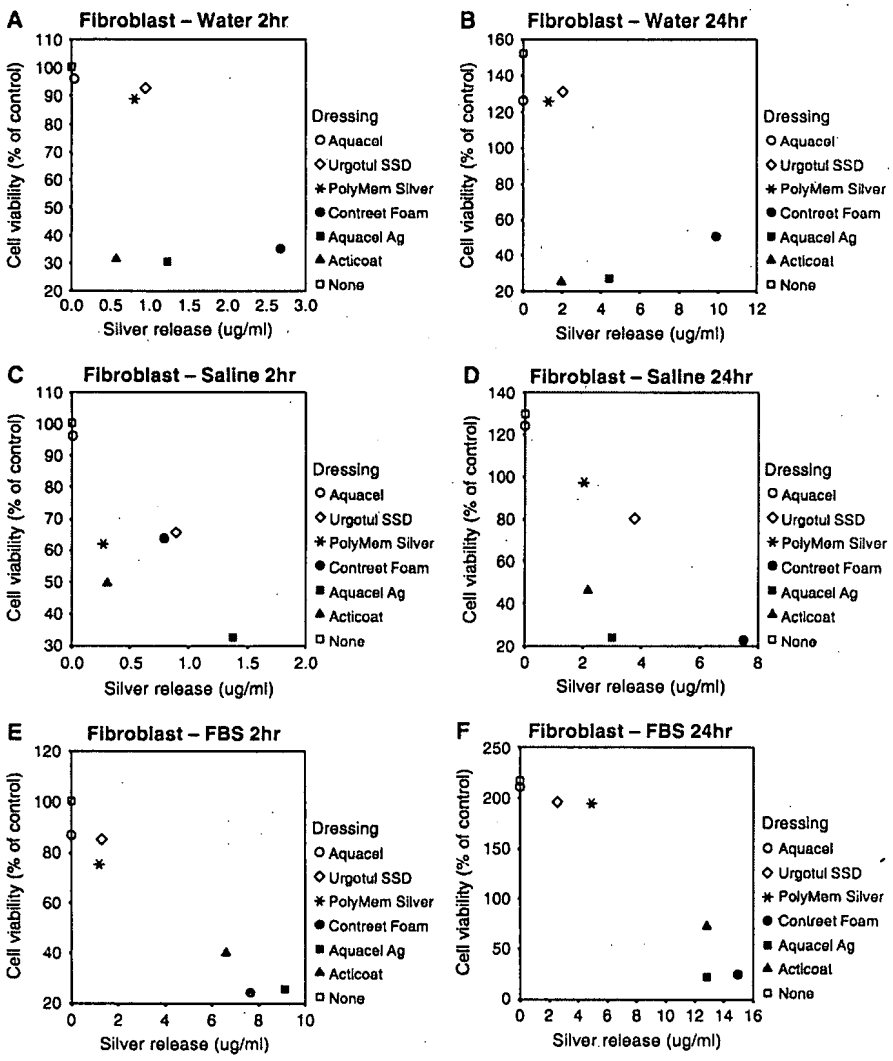

Figure 4. Comparison of the amount of silver released (ug/mL) into fibroblast culture medium with the relative cell viability (%) after various dressing treatments for 2 and 24 hours. The dressings were presoaked with different solutes including water, saline, and fetal bovine serum (FBS; see details in "Materials and Methods"). The total amount of silver released into the culture medium over time was determined by an inductively coupled plasma mass spectrophotometer (ICP-MS) assay. A scatter diagram was plotted to analyze the relationship between the silver concentration and the cell viability.

based dressings remains a significant feature in our clinical application. It was anticipated that the ability of a dressing to exert a significant antimicrobial effect would be directly related to the silver content of the dressing and this was certainly demonstrated in one reported study.[7] The situation appears to be far less clear in the cytotoxicity studies. Thus, for example the measured silver content of PolyMem® Silver was 139 μg/cm^2, which is tenfold higher than that of Contreet® Foam (13 μg/cm^2). Yet, PolyMem® Silver had a less apparent cytotoxic effect than Contreet® Foam. More critical determinants of the potential cytotoxicity of a dressing are the nature of the dressing, in particular, its affinity for moisture as well as the silver composition, that is to say, the distribution of the silver within or on the dressing and the chemical and physical form of the silver (metallic, bound, or ionic). In this study, there are two preparations of nanocrystalline silver: Acticoat™ which delivers the silver from the surface, and, PolyMem® Silver, which is a foam-based dressing. We have demonstrated that PolyMem® Silver has the highest absorbancy among the dressings tested in this study. It also has less silver released into the carrier medium and thus it appears to be "locking up" the silver in the dressing. This is potentially a very good feature of a silver-based dressing where the bacterial "kill zone" is in the dressing rather than in the wound, thus avoiding the "collateral" damage to the healthy cells within the wound.

The test of absorbancy that we have used is based on that described by the State Food and Drugs Administration, China, which in turn is based on / compatible with European reference tests. We have scaled down the amount of material used for cost reasons but in all other respects have found this does give a true and representative comparison with published data. It should be emphasized that we are not aiming to prove or disprove the claims of any commercially sponsored research but rather to look for fair, reasonable, and independent models to evaluate the comparative performance of present and future dressings. The correspondence regarding the validity of testing methodologies is insightful in this regard with the 20-second absorption time in Parson's Convatec-sponsored paper[20] being criticized in Anderson's response (on

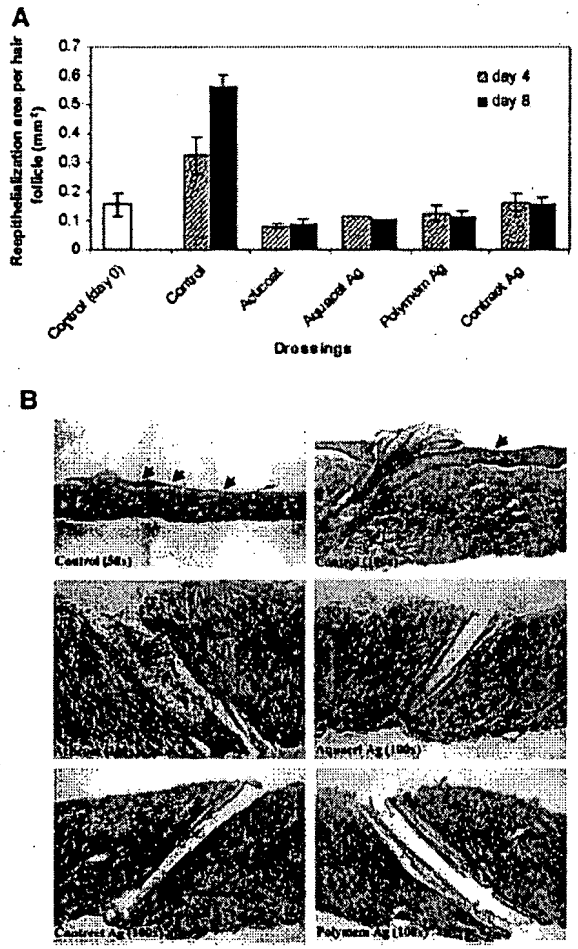

Figure 5. Silver dressings delay epidermal reepithelialization in pig mid-dermis explant culture. (A) The reepithelialization index (reepithelialization area [mm^2] per hair follicle) on days 4 and 8 postdressing delivery was determined (n=3). (B) Examples of histological examination (H&E staining) of cultured tissue explants on day 8 for different experimental treatment groups. The arrows represent reepithelialization areas.

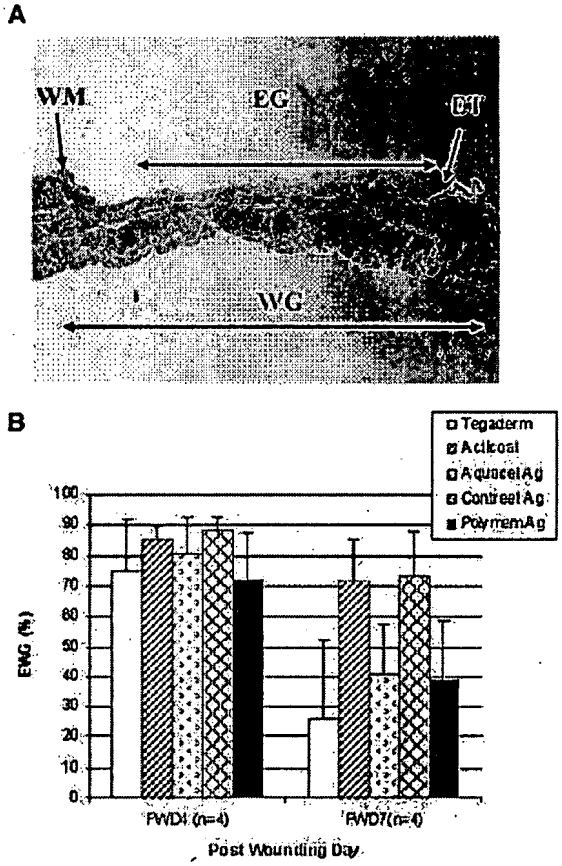

Figure 6. Silver dressings inhibit wound reepithelialization in a mouse excisional wound model. (A) An example of the histological examination (H&E staining) of wound section on postwounding day 4. WM, wound margin; ET, epithelial tongue; EG, epithelial gap; WG, wound gap. (B) Quantitative analysis of wound reepithelialization on postwounding days 4 and 7 for different experimental treatment groups. Data are expressed as the percentage of EG/WG (i.e., EWG %).

behalf of Coloplast).[21] It is also interesting that in the ensuing correspondence, the validity of methodology to determine silver release has also been challenged.

The decision to use a "pretreatment" solution was based on the clinical application of the dressing. According to the manufacturer's instruction, Acticoat™ needs to be moistened with deionzed water before use. Saline is expressly not to be used. Other dressings may be applied dry or moistened. The interaction with the wound will depend upon the fluid environment. For silver to be in a biologically active form, it must be soluble either as Ag^+ or Ag° clusters. Ag° is the metallic or unchanged form of silver found in the nanocrystalline formulations.[9]

What is perhaps surprising is the effect of the pretreatment solution on the cytotoxicity of the various dressings. As an example, Acticoat™ was found to have a significant cytotoxic effect on both keratinocytes and fibroblasts when pretreated with water. Pretreatment with saline significantly reduced the cytotoxicity. The release of silver from Acticoat™ pretreated in saline was significantly reduced when compared with Acticoat™ pretreated with water. The variation in the measured amount of silver released is again surprising. It must be appreciated, however, that the model system is far more complex than that used when manufacturers measure silver release. Thus, the data provided from a Coloplast-sponsored study of in vitro release profiles used a diffusion cell with a continuous flow of 1.4 mL/hour of release media consisting of an isotonic solution with equal amounts of sodium and serum.[22] In our system, the dressings were pretreated and then allowed to equilibrate in two different types of media in which the two cell types were grown. These media are both complex protein-containing solutions.

It was found in the present study that under all test conditions, Aquacel® Ag and Contreet® Foam released a comparable or even greater amount of silver ions than

Acticoat™ at 24-hours postdressing delivery. This, to some extent, was found to lead to a greater cytotoxic effect than Acticoat™. It is also noteworthy that when the dressings were presoaked with FBS, the amount of silver released into both keratinocyte culture medium (K-SFM) or fibroblast growth medium was significantly increased, in particular, in the case of Acticoat™, Aquacel® Ag, and Contreet® Foam. Serum is an extremely complex mixture of plasma proteins, growth factors, hormones, etc. The mechanism involved in serum-promoted silver release is yet unknown, but clearly and generally, the presence of sodium and chloride ions has an effect on silver dissociation.

The pig mid-dermis model is an explant culture model. It provides a three-dimensional system that can also be analyzed in four dimensions (time being the fourth dimension). We have previously used this model to assess the stimulation of cell proliferation and migration by the exogenous application of topical agents.[16] The use of a highly reproducible biological model was appealing and could also provide statistically appropriate comparative data when looking at the area of reepithelialization. In this study we have also compared vertical sections of the explants and selected those that showed clear sections of the hair follicle shaft. We have not elaborated a scoring system for this aspect and so the comparison becomes descriptive rather than statistical. Nevertheless, it is interesting to note that in all four dressings applied, the reepithelialization was not noted. As with other aspects of this current study, there is the potential to explore the biological mechanisms in more detail for example using immunohistochemical staining of matrix protein. This is, however, beyond the scope of this paper. The mouse wound-healing model again focuses on reepithelialization. The typical wound would quickly heal by contraction so the silicone splint keeps it open to allow closure by reepithelialization to be measured. Both models demonstrated delayed or inhibited reepithelialization by silver-based dressings. Taken together, our findings may explain the clinical observation of delayed wound healing or inhibition of wound epithelialization after the use of topical antimicrobial dressings. We suggest that silver-based dressings should be used with caution in situations where rapidly proliferating cells may be harmed as in donor sites, superficial burns, and application of cultured cells. It must be observed that previous studies that demonstrate the silver enhances acute wound healing were performed on incisional wounds where keratinocyte proliferation is not a major feature.[23]

Silver dressings are used in a wide range of wound-healing situations. In our clinical practice, we are more involved in the acute wounds and the emphasis is more on the prevention of infection and promotion of healing. It was the study of the clinical data pertaining to Acticoat™ and reepithelialization that caused our initial questioning of the validity of such studies. On the one hand, Innes et al. demonstrated that nanocrystalline silver impedes reepithelialization in donor sites,[24] while Demling and DeSanti were demonstrating that it increases the rate of reepithelialization in meshed skin grafts.[25] Is one or another of these studies flawed or is there an explanation for both to be correct?

We also have to recognize the clinical anomalies of positive cultures being grown from wounds that have been dressed with silver-based products. This cannot simply be attributed to silver resistance, which is in fact quite rare.[26] Rather, it may well be that the bacteria in vivo behave differently from the bacteria in vitro such that silver is not such an effective killer in the wound as it is in the laboratory.

The requirements of a dressing in a chronic wound-healing situation are different where the control of the wound bioburden is more important.[27] Of course, this was one of the strengths of the hypochlorite solutions that ran into disrepute because of their laboratory-based toxicity.[15] It is a concern when undertaking a study of commercially available materials that results will be quoted out of context. It is certainly not our intention to endorse or criticize any specific dressings. Rather, it has been our intention to explore the variability of the performance in the model systems described and thereby gain a greater understanding of the potential biological interactions of silver and wounds. It is important to keep in vitro derived data in perspective but this applies not only to the cell cytotoxity of silver products but also their ability to effectively deliver silver and kill bacteria in vivo. Ultimately, the best evidence to support clinical effectiveness will come from randomized prospective blinded studies. Such comparative studies are awaited.

ACKNOWLEDGMENT

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Exhibit N

AG107

Agar, Flake, NF

CAS 9002-18-0

125 g 29.50
500 g 86.55
2.5 kg 355.65

Botanic Characteristics ..... As required

MAXIMUM LIMITS

Microbial Limit	To pass test
Water	20.0%
Total Ash (Dried Basis)	6.5%
Acid-Insoluble Ash (Dried Basis)	0.5%
Foreign Organic Matter	1.0%
Foreign Insoluble Matter	1.0%
Arsenic (As)	3 ppm
Lead (Pb)	0.001%
Heavy Metals	0.004%
Foreign Starch	To pass test
Gelatin	To pass test
Water Absorption	To pass test
Organic Volatile Impurities	To pass test

AG105

Agar, Granular, NF

CAS 9002-18-0

125 g 34.05
500 g 97.95
2.5 kg 376.85

Specifications: Same as AG107, p. 27

AG110

Agar, Powder, NF

Agar is the dried, hydrophilic, collodial substance extracted from *Gelidium cartilagineum* (Linne) Gaillon (Fam. *Gelidiaceae*), *Gracilaria confervoides* (Linne) Greville (Fam. *Sphaerococcaceae*), and related red algae (*Class Rhodophyaceae*).

CAS 9002-18-0

125 g 29.50
500 g 86.55
2.5 kg 355.65
25 kg ..... See Bulk Catalog

Specifications: Same as AG107, p. 27

1. Additional carrier-imposed hazardous material charge.

A1672
Agar, Powder, FCC
A dried hydrophilic, colloidal polygalactoside extracted from *Gelidium cartilagineum* (L.) Gaillon (Fam. *Gelidiaceae*), *Gracilaria confervoides* (L.) Greville (Fam. *Sphaerococcaceae*), and related red algae (Class *Rhodophyceae*).
CAS 9002-18-0

125 g 29.50
500 g 86.55
2.5 kg 355.65

MAXIMUM LIMITS

Arsenic (as As)	3 mg/kg
Ash (Acid-Insoluble)	0.5%
Ash (Total)	6.5%
Gelatin	To pass test
Insoluble Matter	1.0%
Lead (Pb)	5 mg/kg
Loss on Drying	20.0%
Starch	To pass test
Water Absorption	To pass test

A3953
Agaric Acid
[Agaricic Acid; 2-Hydroxy-1,2,3-nonadecanetricarboxylic Acid]
Has been used as antiperspirant.
$C_{22}H_{40}O_7$ F.W. 416.55 CAS 666-99-9

250 mg 60.80
1 g 152.00
5 g 505.00

STORE IN A COOL PLACE

A3572
Agarose, -0.13 EEO, Ultrapure
[Amresco Agarose I*]
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

10 g 46.55
25 g 94.85

Appearance	Off-white powder

MAXIMUM LIMITS

Loss on Drying	10%

A3627
Agarose, -0.17 EEO, Ultrapure
[Amresco Agarose I*]
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 204.40

Gelling Point (2.0%)	36°C
EEO	-0.17
Sulfate (SO_4)	0.35%

A1059
Agarose, High Gel Temperature, Electrophoresis Grade
[Amresco Agarose I*]
Applications: Nucleic acid electrophoresis; other procedures where very low electroendosmosis is desired.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 92.40
100 g 285.65

Appearance	To pass test
Gel Temperature (1.5%)	36°-39°C

MAXIMUM LIMITS

EEO	0.1
Sulfate (SO_4)	0.30%
Ash	0.5%
Moisture	10.0%
DNase, RNase and Protease	None detected

AG115
Agarose, Low Electroendosmosis, Electrophoresis Grade
[Amresco Agarose I*]
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 81.10
100 g 250.20

Gel Point	Approx. 36°C
Gel Strength, 1% @ 36°C	Min. 800 g/cm²

MAXIMUM LIMITS

EEO	0.15
Sulfate (SO_4)	0.35%

A3633
Agarose, Low Electroendosmosis, Low Sulfate
[Amresco Agarose I*]
Suitable for use in high resolution electrophoresis of serum plasma, cerebrospinal fluid and urine.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 54.55
100 g 187.50

MAXIMUM LIMITS

Sulfate (SO_4)	0.20%
Water (KF)	7.0%

A3638
Agarose, Medium Electroendosmosis
[Amresco Agarose I*]
Gelling agent for electrophoresis of macromolecules.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 75.50
100 g 228.95

Melting Point	Approx. 260°C

MAXIMUM LIMITS

Sulfate (SO_4)	0.25%

A3168
Agarose, Superfine Resolution, Electrophoresis Grade
[Amresco Agarose I*]
Same ability as acrylamide to resolve DNA fragments less than 1,000 bp. Suitable for the analysis of AMPFLP's, STR's and transnucleotide repeats.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 177.85
100 g 438.60
250 g 1060.20

Gel Strength (1.5%)	Min. 860 g/cm²
Melting Range (1.5%)	65°-69°C
pH of a 1% Solution in Water @ 25°C	As reported

MAXIMUM LIMITS

Gelling Temperature (1.5%)	30°C
EEO (-mr)	0.13
Sulfate (SO_4)	0.075%
DNase, RNase and Protease	None detected

Try our Trismat* brand of Tris [Tris(hydroxymethyl) aminomethane] for your biological buffer needs.
Please see pg. 1149

*Trademark. See trademark index for ownership.

A3165
Agarose, High Resolution, Electrophoresis Grade
[Amresco Agarose I*]
Provides high resolution of small nucleic acid fragments and PCR* products.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 118.50
100 g 355.55
250 g 725.65

Appearance	Fine, white powder
Gel Strength (1.5%)	Min. 2,000 g/cm²
Gelling Range (1.5%)	34°-38°C
Melting Range (1.5%)	85°-89°C
MAXIMUM LIMITS	
EEO	0.12
Sulfate (SO_4)	0.10%
DNase, RNase and Protease	None detected
Function Test	To pass test
Endonuclease / Ligase Inhibitory Factors	None

A3171
Agarose, Type I, Electrophoresis Grade
[Amresco Agarose I*]
Suitable for a wide variety of nucleic acid and protein analytical applications.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

25 g 75.65
100 g 286.30
250 g 602.10

Gel Strength	Min. 1200 g/cm²
Gelling Range	36°-39°C
Melting Range	87°-89°C
MAXIMUM LIMITS	
EEO	0.10
Sulfate (SO_4)	0.15%
DNase, RNase and Protease	None detected

2120 and 2125
Agarose, OmniPure*
For Molecular Biology
Suitable for a wide range of nucleic acid and protein gel applications. Of average gel strength and standard melting and gelling ranges.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 342.80
500 g 1167.55

Appearance	Fine, white powder
Gel Strength (1.0%)	1200 g/cm²
Gelling Range (1.0%)	36°-39°C
Melting Point (1.0%)	87°-89°C
MAXIMUM LIMITS	
EEO (-mr)	0.10
Sulfate (SO_4)	0.15%
DNase, RNase and Protease	None detected

2090
Agarose, High Gel Strength, OmniPure*
For Molecular Biology
For pulsed field applications. Can also be used for resolving nucleic acids down to 200 bp.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 242.65

Appearance	White powder
Gel Strength (1.5%)	3200 g/cm²
Gelling Range (1.5%)	36°-37.5°C
Melting Point (1.5%)	87°-89°C
MAXIMUM LIMITS	
Ash	0.25%
EEO (-mr)	0.12
Sulfate (SO_4)	0.12%
Moisture	7.0%
DNase, RNase and Protease	None detected

2070
Agarose, Low Melting, OmniPure*
For Molecular Biology
Preparative agarose that melts at 65°C and remains liquid at 37°C for several hours.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 668.35

Appearance	White powder
Gel Strength (1.0%)	250 g/cm²
Gelling Range (1.0%)	27°-29.5°C
Melting Point (1.0%)	62°-68°C
MAXIMUM LIMITS	
EEO (-mr)	0.15
Sulfate (SO_4)	0.15%
DNase, RNase and Protease	None detected

2010
Agarose, PCR Plus, OmniPure*
For Molecular Biology
Especially for resolution of small DNA fragments of less than 1000 bp and PCR products. Prevents smearing or high fluorescence backgrounds.
$[C_{12}H_{14}O_5(OH)_4]_n$ CAS 9012-36-6

100 g 342.80
500 g 1167.55

Appearance	Fine, white powder
Gel Strength (1.5%)	2000 g/cm²
Gelling Range (1.5%)	34°-38°C
Melting Point (1.5%)	85°-89°C
MAXIMUM LIMITS	
EEO (-mr)	0.12
Sulfate (SO_4)	0.10%
Endonuclease/Ligase Inhibitory Factors	None detected
DNase, RNase and Protease	None detected

AGGC See N-Acetyl-S-geranylgeranyl-L-cysteine, p. 16